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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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BIOLOGICAL BULLETIN

RESPIRATORY METABOLISM.

PHYSIOLOGICAL STUDIES ON RESPIRATORY METABOLISM.[1]

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INTRODUCTION.

Such quantitative knowledge as now exists concerning the fruit fly, *Drosophila*, has been largely obtained from genetical studies by Morgan and his students (1). Quantitative physiological studies on this organism, however, have been carried out principally by Loeb and Northrup (2) and more recently by Pearl and his associates (3). These authors have been concerned primarily with studies on the duration of life of the organism under normal as well as experimental conditions. Inasmuch as no quantitative information seems to exist concerning such questions as the physiological differences between stocks or strains, the physiological differences between sexes, etc., it was deemed advisable to study these questions in some detail. The present paper is one of a series dealing with certain physiological phenomena such as, first, changes in the oxygen and carbon dioxide content of culture bottles; secondly, the rates of carbon dioxide output; thirdly, the rates of oxygen consumption of two different stocks or strains of animals, the so-called 'wild' and 'vestigial' types.

MATERIAL AND METHODS.[2]

Two different stocks or strains of *Drosophila melanogaster,* the so-called "wild" and "vestigial" types, were used in these experiments. These stocks were obtained from Dr. R. L. King of this laboratory and have been bred continuously by him for many generations. The vestigial stock is a synthetic one carrying two second chromosome mutations, vestigial and black, in homozygous form.

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[1] Hereditary differences in respiratory metabolism of two stocks of *Drosophila.*

[2] Parts of the data in this paper are taken from a thesis submitted by P. R. O. to the graduate school of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Master of Science.

The stocks were carried along in the laboratory at room temperatures in quart milk bottles and fed the usual banana-agar-

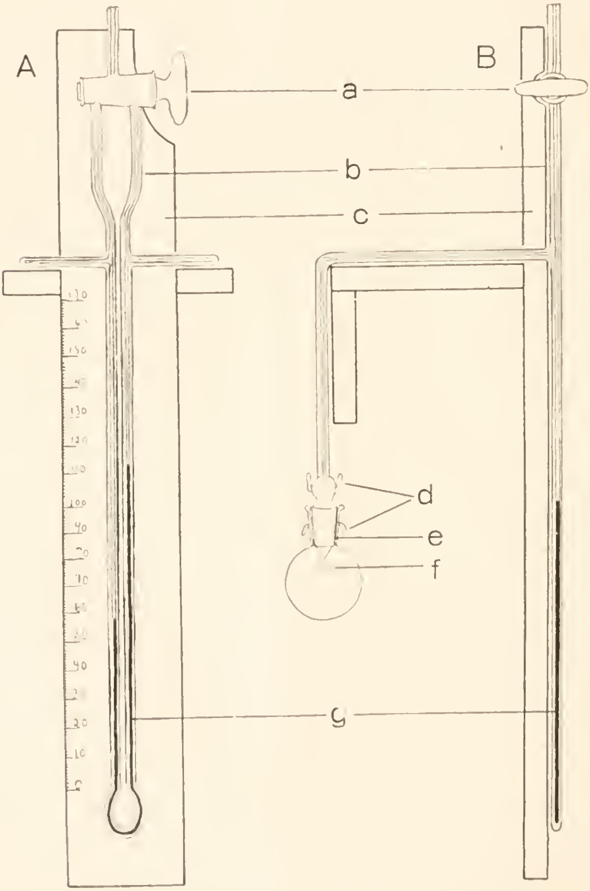

FIG. 1. Diagram of modified Krogh micro-respiration apparatus. *A*, front view. *B*, side view. *a*, glass-ground stopcock; *b*, capillary tubing with bore of approximately 0.4 mm. in diameter; *c*, wooden stand for supporting manometer; *d*, glass hooks for holding respiration chamber to manometer by means of elastic bands; *e*, glass-ground joint; *f*, glass spoon support for organism; *g*, kerosene in manometer.

Note that entire manometer is made of glass with ground connections.

yeast mixture as used by geneticists and others working with flies (Pearl 3). The experimental animals were kept in 100 cc. culture bottles in an electric incubator at 25° C. Small square pieces of filter paper were put in the culture bottles so that the larvæ could crawl up on them and pupate. The filter paper with the attached pupæ were removed at regular intervals and pupæ of known age thus obtained. The pupæ were carefully removed from the filter paper, cleaned and washed, then sterilized in 95 per cent. alcohol and again washed. They were then put into small shell vials containing small pieces of moistened filter paper to prevent drying. The vials were marked, stoppered with a small cotton plug and kept in the incubator at 25° C. Great care is necessary in handling individual pupæ because of their small size and the fact that they are easily injured. A very thin metal spat-

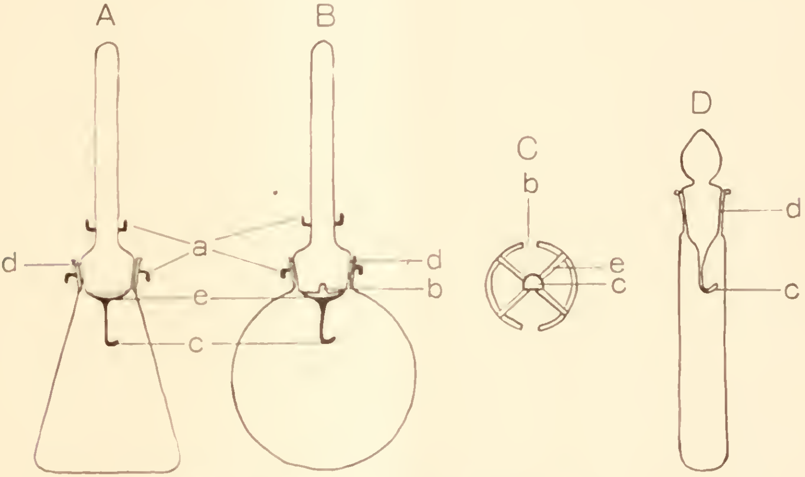

Fig. 2. Diagrams of different types of apparatus used in CO_2 determinations. *A*, flask of approximately 250 cc. capacity, which can be used either as a standard or as a respiration chamber. *B*, same as *A*. *C* shows attachment of animal holder (*e*) as well as trough (*b*). *D*, respiration chamber, 3 cc. capacity. *a*, glass hooks for holding parts of flask together by means of elastic bands; *b*, trough in glass to allow indicator solution to run from flask to attached comparing tube without coming in contact with supports of animal holder or spoon; *c*, glass animal holder or spoon; *d*, glass-ground joints; *e*, glass supports for animal holder or spoon. Apparatus is made entirely of Pyrex or Non-sol glass with no rubber stoppers or connections. Joints are all ground.

ula has been found a most convenient instrument for handling the organisms. In weighing pupæ a delicate analytical balance was used.

Since the purpose of the present investigation was primarily to determine the rates of oxygen consumption and carbon dioxide output of the different stocks of flies, it was necessary to have methods accurate and delicate enough to measure the respiratory exchange of such small organisms. The following types of apparatus were found to fulfill these requirements.

For oxygen determinations a modified Krogh (4) micro-respiration apparatus was used. The accompanying diagrams and explanations show the apparatus as finally adopted. For the methods of using the apparatus, derivation of formulæ, etc., the reader is referred to Krogh's (4) complete descriptions and explanations.

Carbon dioxide determinations were made by the Haas (5) indicator method, using suggestions pointed out by Jacobs (6). A decided improvement over the method as suggested by Jacobs, however, is apparatus made entirely of hard glass with no rubber stoppers or connections. The accompanying diagrams and explanations show some of the various types of apparatus employed. For methods of calculating results, etc., with this method the reader is referred to the article by Jacobs (6).

Analyses of the air in culture bottles were made with a Haldane gas apparatus (7), the sample of air being slowly drawn out of the bottle into the apparatus by means of a capillary tube.

Analysis of Air in Culture Bottles.

Pearl and Parker (3) in recent studies on duration of life in Drosophila have pointed out that in the case of wild type, " . . . an increase of roughly 10 per cent. in the mean duration of life is brought about by increasing the ventilation of culture bottles, by covering the mouth with one layer of number 48 mesh bolting cloth as compared with the use of cotton plug stoppers as is the usual practice in cultures of *Drosophila* in the laboratory." Similar experiments were also carried out by these authors using Quintiple type flies and no such differences in duration of life were noted. Inasmuch as these authors report no analyses of the air contained in culture bottles with such stoppers it was thought

TABLE I.

SHOWS AMOUNTS OF CO_2 AND O_2 IN CULTURE BOTTLES STOPPERED WITH VARIOUS TYPES OF STOPPERS FOR DIFFERENT TIME INTERVALS AND ALSO AMOUNTS OF CO_2 AND O_2 IN ROOM IN WHICH BOTTLES WERE KEPT.

Capacity of Bottles—cc.	Time of Analysis after Culture was Begun—Hrs.	Media Used.	Presence or Absence of Flies.	Kind of Stopper.	Analysis, %.			
					Bottle.		Room.	
					CO_2.	O_2.	CO_2.	O_2.
110	24	Normal	+	Cotton plug	0.0114	20.8	0.014	20.86
110	24	"	+	" "	0.018	20.8	0.014	20.86
110	168	"	+	" "	0.05	21.08	0.018	20.5
110	230	"	+	" "	0.052	20.3	0.0103	20.3
110	24	"	+	Gauze plug	0.015	19.6	0.014	20.86
110	24	"	+	" "	0.0154	20.5	0.014	20.86
110	24	"	+	" "	0.0152	20.5	0.014	20.86
110	192	"	+	Gauze stretched over top	0.0518	21.59	0.016	20.5
110	360	"	+	" " " "	0.053	20.4	0.0103	20.3
110	192	"	+	Wire gauze stretched over top	0.0426	20.9	0.016	20.5
110	192	"	+	" " " " "	0.037	21.2	0.016	20.5
110	360	"	+	" " " " "	0.0309	20.1	0.0107	20.3
110	168	"	+	Gauze stretched over top	0.058	21.8	0.018	20.5
110	192	"	+	" " " "	0.0375	20.3	0.016	20.5
110	192	"	+	" " " "	0.032	21.8	0.016	20.5
75	120	"	+	Glass	61.2	5.3	0.018	20.5
75	120	"	+	"	53.09	5.35	0.018	20.5
110	192	"	+	Cork	41.9	9.03	0.018	20.4
110	288	"	+	"	55.4	4.1	0.018	20.4
75	288	None	+	Glass	2.17	16.2	0.018	20.4
110	288	Wet filter paper	+	Gauze stretched over top	0.015	21.03	0.018	20.4
75	282	" " "	+	Glass	3.9	16.3	0.018	20.4
110	48	Yeast and banana	—	Gauze stretched over top	0.016	21.6	0.018	20.4
110	48	" " "	—	Cotton plug	0.026	21.2	0.018	20.4
75	24	" " "	—	Glass	1.06	18.15	0.016	20.5
75	168	" " "	—	"	Gas formed; blew stoppers out.			

desirable to a least make accurate quantitative analysis of the carbon dioxide and oxygen content of the culture bottles used in the present experiments.

Culture bottles were variously stoppered as indicated in Table I. and analyses made of their carbon dioxide and oxygen contents. In making the analysis the air samples were at first taken from different levels in the bottles beginning just below the stopper and finally just above the medium. No marked differences in the carbon dioxide and oxygen content of the air samples from different levels in the bottles were noted except that when the sample was taken at the surface of the medium slight differences in amounts of the two gases were usually found. All samples used and indicated in Table I. were taken from the middle of the culture bottles.

An examination of this table shows that in bottles with cotton plugs, gauze plugs, gauze stretched over the tops, etc., there were no appreciable differences in the amounts of carbon dioxide and oxygen noted. In bottles stoppered with cork or glass rather marked increases in the carbon dioxide content and decreases in the oxygen content were found. It must be pointed out here, however, that in these experiments only carbon dioxide and oxygen have been measured and that no account of other gases formed has been taken into consideration.

Behavior of Flies in Differently Stoppered Bottles.

The behavior of flies kept in differently stoppered bottles as noted by Pearl and Parker (3) is of considerable interest. These authors found that in bottles stoppered with gauze the wild flies tended to congregate at the top of the bottle under the bolting cloth while no such behavior was noted in the case of the Quintiple flies. This behavior in the case of the wild flies was not noted in bottles stoppered with cotton. The increased amounts of fresh air in the case of the bottles stoppered with gauze is given by these authors as the cause of this behavior.

Experiments were carried out by the present authors to test the behavior of wild flies in differently stoppered culture bottles (capacity 100 cc.) but no significant differences were noted As a matter of fact if a cylinder was made of wire gauze the same size as the culture bottles, and the two ends covered with white cloth gauze, the flies tended to congregate under the gauze cover when cylinder was placed in an upright position despite the fact that the composition of the air in the cylinder was uniform throughout. By changing the position of the culture bottles, as well as of the wire gauze cylinder, in respect to the source of light the flies could be made to congregate at either end whether cotton plugs, gauze, etc., were used as stoppers. It seems to the authors that factors other than ventilation, doubtless light, are responsible for such behavior of the flies in differently stoppered bottles.

Respiratory Metabolism.

Since the genetic constitution of wild and vestigial stocks differs greatly, it was deemed desirable to measure the rates of respiratory metabolism of the two types to see if in this respect equally marked differences could be detected. Such a comparison of respiratory metabolism requires that all body movements, etc., be eliminated and that the organisms be under identical environmental and physiological conditions throughout the experiments. To meet these requirements, in the present investigations, sterilized pupæ, kept under identical conditions, have been used. Since rates of respiratory metabolism are usually expressed per gram body weight per unit of time, it was also necessary to weigh the organisms used in the experiments. The general methods used in obtaining pupal weights were as follows: Pupæ of known age were carefully removed from the filter paper by means of a thin metal spatula, cleaned and washed, then sterilized in 95 per cent alcohol and again washed in water. The excess moisture was next removed by placing the pupæ on dry filter paper. They were then weighed on a delicate analytical balance, in lots of five and ten and the average weight per pupa determined.

Figure 3 based on the weights of several hundred individuals shows graphically the average body weights for pupæ of the two stocks at different ages. It is evident from an examination of this figure that vestigial pupæ are somewhat heavier than wild and remain so throughout their entire pupal life. Vestigials, however, do not produce as many pupæ as the wild. It is necessary, therefore, in experiments to have larger numbers of vestigial cultures for the production of a certain number of pupæ on a particular day than in the case of the wild where the same culture can usually be used for several days. One gets a much larger number of body weights for one day old vestigial pupæ of different cultures than for the wild pupæ where larger numbers of body weights for one day old pupæ are obtained from the same culture. In calculating average body weights, therefore, this fact plays an important part and should be kept in mind especially because of its significance in the determining of the rates of carbon dioxide output and oxygen intake per body weights of the animals. To control adequately such a factor it would doubtless be necessary to change the flies to new culture bottles every day so that the pupæ from the eggs laid in any one day could be obtained. If this factor is taken into consideration and a more extensive study made, we feel certain that a much smaller difference in the average body weights of the two stocks would be noted. It is also of interest to note from Fig. 3 that during the life of the pupæ there is steady decrease in weight. At the emergence of the fly, however, a rather marked increase in weight seems to occur and as a matter of fact the fly just after emerging weighs somewhat more than the pupa from which it came. This increased weight is doubtless due to absorption of water.

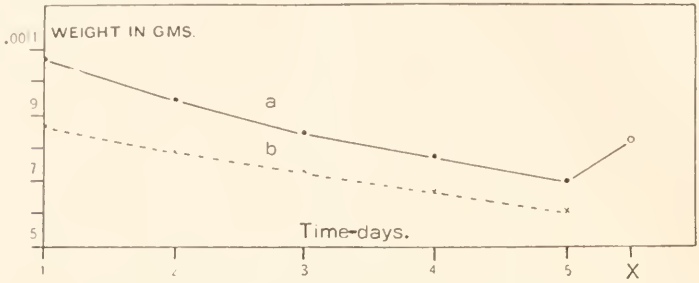

FIG. 3. Shows body weight of wild and vestigial pupæ from day of formation to emergence. Ordinate represents body weight per pupa in grams, abscissa, time in days indicated. X = fly just after emerging; a = vestigial; b = wild.

OXYGEN CONSUMPTION.

Inasmuch as the present paper deals only with a comparison of respiratory metabolism in the two stocks of flies, the data presented here are those obtained by using lots of five and ten pupæ at one time and making determinations daily throughout pupal life. The pupæ in all cases had been under identical environmental conditions and were of the same age. In running through any two series, *i.e.*, wild and vestigial, from the first day to the time of emergence, the same manometer was used for each, so that any possible error in the calibration of instruments would not enter into experiments dealing with the relative respiratory rates of the two stocks.

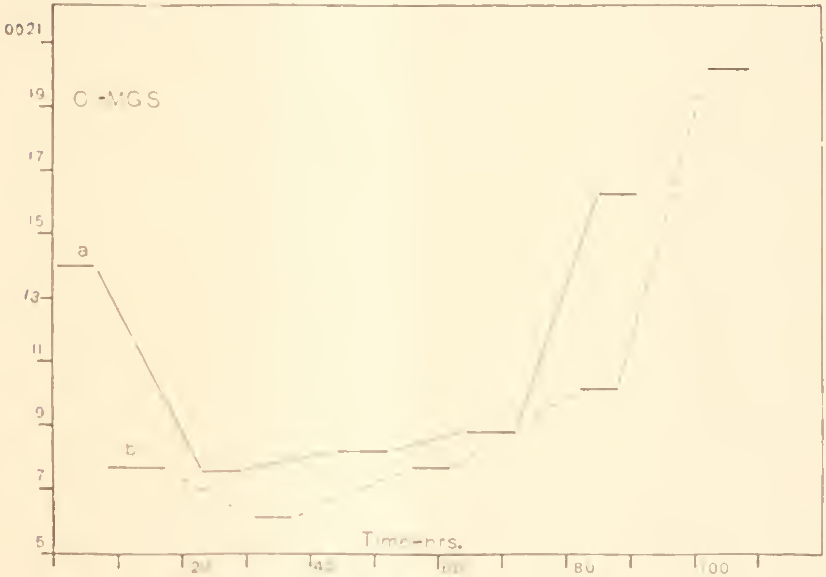

Fig. 4. Shows oxygen consumption (mg.) per mg. body weight per hour for wild and vestigial pupæ during entire pupal life. Ordinate represents oxygen (mg.) per mg. body weight per hour, abscissa time in hours indicated. *a* = wild; *b* = vestigial. Solid horizontal portions of curves indicate time during which oxygen determinations were made.

Table II. and Fig. 4, selected from many experiments, show the oxygen consumption for pupæ of the two stocks. From an examination of these it is evident that the rates of oxygen consumption for the two stocks are quite similar. In both stocks there is a decided decrease in oxygen consumption the second day followed by a gradual increase up to the time of hatching. The rate of oxygen consumption the fifth day usually exceeds that for the first day.

Table II.

Shows Rate of O_2 Consumption for Two Lots of Ten Wild and Ten Vestigial Pupæ during Entire Pupal Life.

Temp. 22.5° C.	Oxygen (mm.³) per Min. per 10 Pupæ.		Oxygen (mg.) per Min. per Pupa.		Oxygen (mg.) per Mg. Body Weight per Min.	
Day of Pupal Life.	Wild.	Vestigial.	Wild.	Vestigial.	Wild.	Vestigial.
1	0.14170	0.09704	0.00002168	0.00001484	0.00002466	0.00001381
2	0.07097	0.06531	0.00001085	0.00000999	0.00001361	0.00001051
3	0.06807	0.07652	0.00001041	0.000011707	0.00001442	0.00001377
4	0.0690	0.09245	0.00001055	0.00001414	0.00001582	0.00001826
5	0.11780	0.16630	0.00001802	0.00002544	0.00002921	0.00003634

Table III.

Shows O_2 Consumption for Five Different Lots of Ten Wild and Ten Vestigial Pupæ during Entire Pupal Life.

Day of	Oxygen (mm.³) per Min. per Pupa	
Pupal Life	Wild	Vestigial
1	0.010146	0.008362
2	0.004531	0.007446
3	0.006699	0.011182
4	0.008459	0.014291
1	0.010020	0.010240
2	0.006434	0.007302
3	0.008194	0.008917
4	0.008892	0.016470
1	0.009909	0.008321
2	0.007426	0.008266
3	0.006790	0.011222
4	0.007846	0.011496
5	0.009470	0.013412
1	0.008200	0.015240
2	0.008449	0.007724
3	0.011587	0.011340
4	0.019070	0.017620
1	0.007405	0.009390
2	0.006730	0.006340
3	0.008660	0.010725
4	0.010870	0.011960

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[3] It is a pleasure to acknowledge receipt of a grant from the Joseph Henry Fund of the National Academy of Science for the purchase of a sensitive water thermostat for this work.

Table III. shows results for five different lots of ten wild and ten vestigial throughout pupal life and also gives some idea of the range of variation existing in animals of the two types.

Carbon Dioxide Output.

Ten pupæ were used at one time and determination of carbon dioxide output made on successive days from the first day of pupation till hatching as in the case of oxygen determinations.

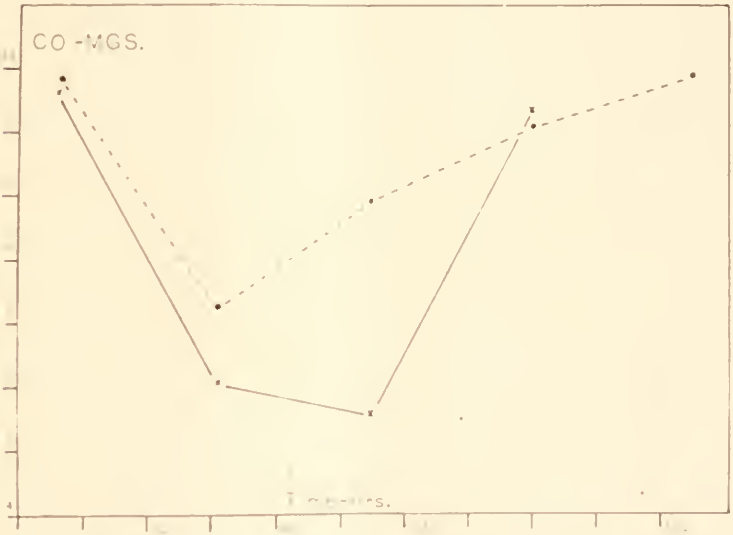

Fig. 5. Shows carbon dioxide output (mg.) per mg. body weight per hour for wild and vestigial pupæ during entire pupal life. Ordinate represents CO_2 (mg.) per mg. body weight per hour, abscissa time in hours indicated. *a* = vestigial; *b* = wild.

Table IV.

Shows Rate of CO_2 Production per Mg. Body Weight per Minute for Two Lots of Ten Wild and Ten Vestigial Pupæ during Entire Pupal Life.

Day of Pupa Life.	CO_2 (mg.) per mg. Body Weight per min. Wild.	Vestigial.
1	0.00002704	0.00003052
2	0.00001025	0.00001230
3	0.00000934	0.00001493
4	0.00002197	0.00001840
5		0.00003321

1	0.00003129	0.00005140
2	0.00001462	0.00001928
3	0.00001465	0.00002017
4	0.00001583	0.00002759
5	0.00003942	0.00009972

Table IV. gives the amounts of carbon dioxide produced by ten wild and ten vestigial pupæ during their entire pupal life. Fig. 5 also shows graphically the rates of carbon dioxide production for two lots of pupæ. From an examination of this figure it will be noted that the curves follow closely those for oxygen consumption with characteristic decreases the second day, followed by steady increases up to the time of hatching. Vestigials seem to have the higher rate of carbon dioxide production.

Table V.

Shows Time Required to Produce the Same Amount of CO_2 by Ten Different Groups of Ten Wild and Ten Vestigial Pupæ during Entire Pupal Life.

Day of Pupal Life.	Time to Produce Same Amount of CO_2—Minutes.			
	Wild.	Vestigial.	Wild.	Vestigial.
1	4	3	7	5
2	10	8	12	11
3	8	6	11	8
4	5	4	4	6
5	3	2		3
1	5	4	4	3
2	11	9	9	7
3	10	7	8	5
4	8	5	3	2
5	4	3		
1	3	2	6	4
2	10	6	7	8
3	8	4	7	6
4	2	1.5	3	2
1	4	3	6.5	5.75
2	8	7	10	8
3	7	6	9.5	7
4	6.5	4	2.3	5.3
5	2	1.5		2
1	5	3.5	1.5	1.3
2	8	9	6.7	5
3	8	8	4.8	4.5
4	3	7	2.8	4
5		2		2.3

Table V. gives examples of the variations in carbon dioxide production in ten different lots of ten wild and ten vestigial pupæ. A rather wide range of variation will be noted in the time taken by different lots of the same age to produce the same amount of carbon dioxide.

These studies are being continued in this laboratory, with the idea of investigating, in a quantitative manner, the possible effects of different factors upon the physiological make-up of the organism, as well as differences due to sex and species.

Summary and Conclusion.

1. No significant differences in air consumption (O_2 and CO_2) in culture bottles with different types of stoppers, all other conditions remaining the same, have been found.

2. There is a gradual decrease in body weight during pupal development of the fly.

3. Rates of oxygen consumption and carbon dioxide output of pupæ follow definitely shaped curves.

4. There is a decrease in oxygen consumption and carbon dioxide output the second day of pupal life followed by a gradual increase up to the time of hatching.

5. A comparison of the rates of respiratory metabolism of two types of flies, wild and vestigial, has been made and more extensive experiments seem necessary before differences found in these stocks can be definitely established.

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THE OCCURRENCE OF *RICKETTSIA*-LIKE MICROÖRGANISMS IN ADULT "LOCUSTS" (*TIBICEN SEPTENDECIM* LINN.).

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While attempting to extend some studies already made dealing with the distribution of *Rickettsia* in insect tissues[1] a number of "locusts" were collected in the vicinity of Teaticket, Massachusetts and were examined microscopically at the Marine Biological Laboratory. Eleven out of 50 of these specimens, or a little over 20 per cent., were found to be heavily infested with peculiar microorganisms which it is the purpose of this note to describe briefly.

In size they resembled *Rickettsia* in being somewhat smaller than most bacteria. After alcoholic fixation and coloration by Giemsa's method they measured from 0.2 to 0.3 micron in diameter and from 0.2 to 1.5 microns in length, with average dimensions of 0.2 by 1.0 micron. They were usually straight with bluntly rounded ends and were sometimes arranged in pairs (Figs. 1–3). Like *Rickettsia*, these microörganisms tended to be stained red by this technique; but unlike *Rickettsia*, they were somewhat resistant to decolorization by Gram's method—this was however a matter of degree in quality rather than one of distinct difference (Fig. 3). They were restricted in distribution to the lumina of the alimentary tract and Malpighian system—that is to say to two locations in which *Rickettsia* are very frequently met with. The manner in which they were seen to be applied in thick masses upon the epithelial surfaces was also suggestive of *Rickettsia* (Fig. 2). They were seldom observed within cells. In infested "locusts" the alimentary and Malpighian systems were not uniformly invaded throughout their whole extent. In serial sections it was a common experience to observe perfectly normal tubules, devoid of microörganisms, and then suddenly to encounter large numbers of them. In these areas the lining epithelium showed no noticeable modifications except a slight reduction in height. Although very careful search was made, none of the microörganisms were detected in the ovaries. Another point of similarity to *Rickettsia* was that the microörganisms did not reveal their presence by causing noticeable sickness in their insect-hosts.

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[1] Cowdry, E. V., *J. Exp. Med.*, 1923, XXXVII., 431.

Bacteria of larger size and staining blue by Giemsa's method were occasionally observed in small numbers within the body cavities of the same insects; but not in the intestinal lumina, or within the Malpighian tubules. Some of these bacteria are shown in the lower part of Fig. 2.

The alimentary tract, though well developed, was never distended with food but contained a small amount of a coagulum which might well have been produced by the action of enzymes and fixatives upon plant juices—accepting the evidence of Quaintance,[2] Snodgrass,[3] and others that the insects take food of this kind. Hargitt,[4] on the other hand, is of the opinion that the insects seldom if ever feed in the adult state. If substantiated, Hargitt's view will narrow down a search for the port of entry of the parasites.

The term "*Rickettsia*" has been applied to an ill-defined group of microörganisms which are difficult to classify with certainty owing chiefly to their resistance to ordinary methods of artificial cultivation. It was first used by da Rocha Lima,[5] in honor of Doctor H. T. Ricketts, who died while investigating typhus fever, to designate certain peculiar microörganisms which he found in coöperation with Doctor R. M. Wilder.[6] Since that time the word has gradually become generic in its scope and is now generally conceded to include also the parasite of Rocky Mountain spotted fever, certain organisms held to be responsible for trench fever and Japanese River sickness, as well as many which are not known to be pathogenic for man. About 25 different varieties of *Rickettsia* have thus far been reported. According to Wolbach,

[2] Quaintance, A. L., Bull. No. 87, Maryland Agr. Exp. Sta., 1902.

[3] Snodgrass, R. E., Smithsonian Report for 1919, published 1921, 381.

[4] Hargitt, C. W., Biol. Bull., 1923, XLV., 200.

[5] da Rocha Lima, H., *Berl. klin. Wchnschr.*, 1916, LIII., 567.

[6] Ricketts, H. T., and Wilder, R. M., *J. Am. Med. Assn.*, 1910, LIV., 1304, 1373.

Todd, and Palfrey:[7] "In every instance where careful study has been made it has been found—with the exception of the rickettsia of typhus—that the organisms pass down through successive generations in the eggs."

It is in connection with this possibility of hereditary transmission that further study of the microörganisms mentioned in this note may be interesting, particularly since their hosts exhibit a very unusual life history, some aspects of which have been made the subject of recent contributions to the BIOLOGICAL BULLETIN by Hargitt[4] and by Hickernell.[8] The particular Massachusetts brood, with which we are concerned, is possessed of a distinguished ancestry. Marlatt[9] quotes a description of the first swarm noted by settlers in 1634, in Moreton's words, as follows:

"It is to be observed that the Spring before this Sickness, there was a numerous company of *Flies*, which were like for bigness unto *Wasps* or *Bumble-Bees*, they came out of little holes in the ground, and did eat up the green things, and made such a constant yelling noise as made all the woods ring of them, and ready to deaf the hearers; they were not any of them heard or seen by the *English* in the country before this time: But the *Indians* told them that sickness would follow, and so it did . . . ."

By citing this historic reference and by mentioning certain points of resemblance between the microörganisms and *Rickettsia*, the implication is not intended that the "locusts," or their parasites, are in any way associated with human disease. During the great "locust" plague of 1868 and during every important reappearance of the insects before and since that time there have been many reports of the infliction of more or less serious stings. We have good authority (Riley) for assuming, however, that these stings are not caused by the "locusts" themselves but by other insects, such as digger wasps, which are often prevalent at the same time.

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[7] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., "The Etiology and Pathology of Typhus." Harvard University Press. Cambridge, Mass., 1922, 222 pages.

[8] Hickernell, L. M., BIOL. BULL., 1923, XLV., 213.

[9] Marlatt, C. L., "The Periodical Cicada." Bull. No. 71, Bureau of Entomology, Washington, 1907, 181 pages.

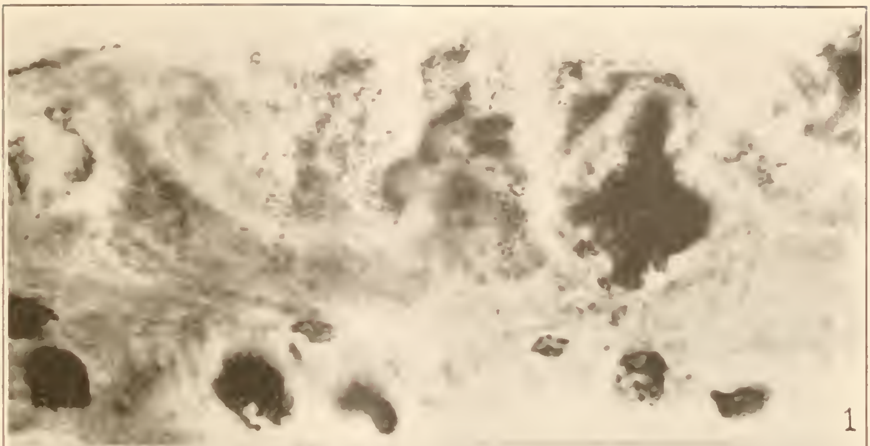
Returning to our Massachusetts brood, there is a gap in our records from 1634 to 1789 but published accounts [9] report the reappearance of the insects every 17 years from 1789 to 1923. There is every reason to believe that they have, with the utmost regularity, maintained this 17-year cycle for upwards of 300 years, with the prospect of continuing indefinitely, or until they are killed out by the removal of underbrush. Undoubtedly the *Rickettsia*-like microörganisms, to which attention has been called, have selected very remarkable hosts.

EXPLANATION OF PLATES.

FIG. 1. Intestine of adult "locust," alcoholic fixation, Giemsa's stain, and photograph at 1,000 diameters.

FIG. 2. Malpighian tubule of adult "locust," same fixation and staining, photograph at 1,000 diameters.

FIG. 3. Intestine of adult "locust," alcoholic fixation, Gram's stain, and photograph at 1,500 diameters.



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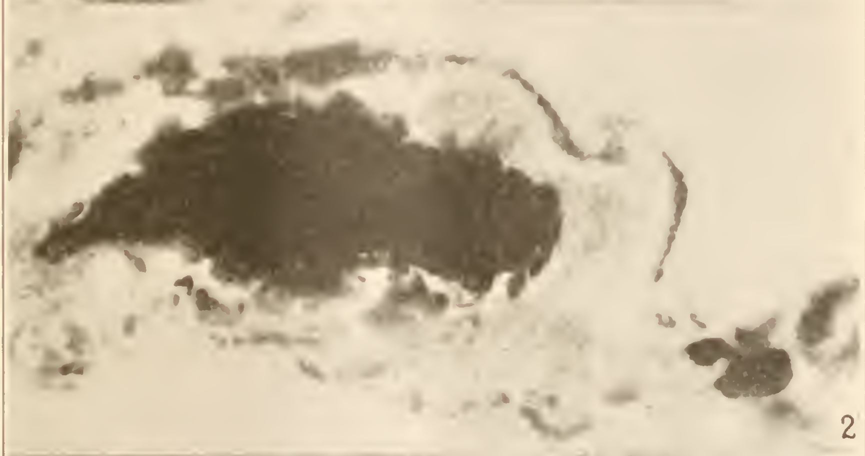

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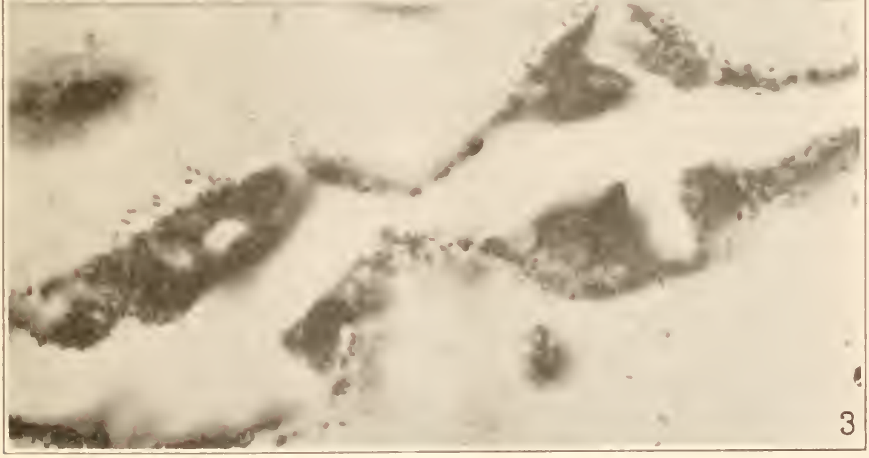

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E. V. COWDRY.

THE AXIAL GRADIENTS IN HYDROZOA.

VI. EMBRYONIC DEVELOPMENT OF HYDROIDS.

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During the summers of 1917 to 1920, spent at the Puget Sound Biological Station, Friday Harbor, Washington, some time was spent in the study of polarity in eggs and embryos of several species of hydrozoa and of modification and control of development through differential susceptibility. Continuation, of the work has not been possible since 1920, but the data already at hand are consistent in themselves and constitute further evidence for the existence and importance in development of the physiological gradients as well as a basis for interpretation in physiological terms of the transformation of the planula into the hydroid form. This opportunity is taken to acknowledge again my obligations to the Director of the Puget Sound Biological Station for the facilities placed at my disposal.

Material and Methods.

Because of its abundance the hydromedusa, *Phialidium gregarium* constituted the chief source of embryonic material. Since the medusæ shed eggs and sperm more or less continuously in small quantities, fertilized eggs were most readily obtained in quantity by keeping medusæ in considerable numbers, often several hundred, in large pans or tubs for a few hours and then collecting eggs and embryos from the bottom. In such material stages may range from newly shed eggs to various cleavage stages or early blastulæ, according to the length of time the medusæ have been in the container. Embryonic stages of *Stomotoca atra* were obtained in the same way, but the medusæ of this species were usually much less abundant than *Phialidium*. Ovarian eggs of all stages of growth were obtained directly from the ovaries of these species. In a third species, *Gonothyræa clarkii,* which was used to some extent, the free swimming medusa stage is absent, the early embryonic development occurring in gonophores on the hydroid. Planulæ of this species were obtained by keeping the gonophore-bearing hydroids in the laboratory for a few days and earlier stages, by opening the gonophores. The development of *Phialidium* and *Gonothyræa* was followed through the transformation of planula into hydroid stage, that of *Stomotoca* only to the late planula. *Phialidium* and *Gonothyræa* give rise to campanularian hydroids; *Stomotoca* is an anthomedusa.

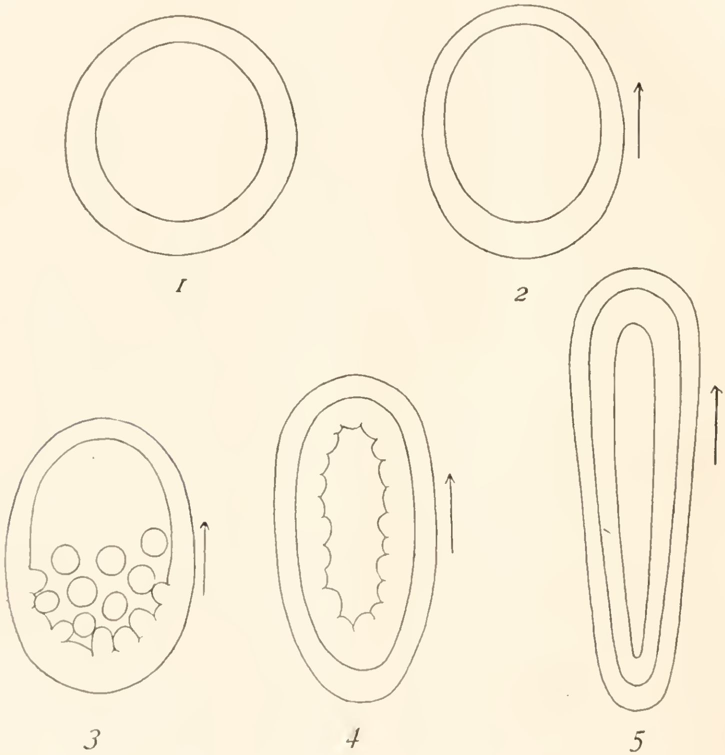
The axial physiological gradients in eggs and developmental stages were demonstrated as susceptibility gradients by disintegration or cytolysis in KCN in indicated concentrations, *m*/100 to *m*/500 in sea water, $HgCl_2$ in indicated concentrations, *m*/50,000 to *m*/500,000 in sea water, methylene blue and neutral red in low concentrations in sea water; second, as gradients in rate and amount of reduction of $KMnO_4$ in various low concentrations in sea water and in rate of reduction of methylene blue; third, as gradients in rate of penetration of the vital dyes, neutral red and methylene blue. The concentrations of agents used are given as "indicated concentrations" because the normal medium sea water is used as solvent in all cases and the concentration given is merely that indicated by the amount of the agent which is added to a certain volume of sea water. As regards $KMnO_4$ and the vital dyes. the same results are obtained with a wide range of concentrations because these substances are taken up from very low concentrations and accumulate in unchanged or reduced form in the protoplasm. The point of chief importance is that the concentration be low enough so that the staining or disintegration by the dyes and the reduction of $KMnO_4$ shall not occur so rapidly as to obscure the differences at different levels.

A $KMnO_4$ solution, indicated concentration *m*/1,000, served as stock solution and was diluted two to five times or even more for use in demonstrating differences in rate of reduction. Frequently a drop or two of the stock solution was added from time to time as reduction decreased the concentration. Very low concentrations of $KMnO_4$ *e.g.*, below *m*/10,000, produce more or less cytolysis and can be used like other cytolytic agents to demonstrate the gradients. Special attention may be called to the fact that the permanganate gradient is not simply a gradient in the rate of reduction of $KMnO_4$ but when the reaction proceeds to completion in excess of $KMnO_4$ a gradient in depth of color indicating differences in amount of reduction is evident. For demonstration of these differences the material is kept in excess of $KMnO_4$ solution 24 to 48 hours and is then dehydrated and cleared since it is opaque in aqueous medium. Such cleared preparations are not permanent in any of the clearing agents thus far used nor in balsam. The brown or blackish color due to MnO_2 or other oxides gradually disappears, apparently by solution, in the course of several days longer. The preparations are sufficiently permanent, however, to permit imbedding and sectioning before the color gradient disappears, though of course fading occurs more rapidly in the sections than in the whole preparations.

For demonstration of the gradients by methylene blue various concentrations, ranging from 1/1,000 to 1/20,000 were used, according to species and result to be attained, the higher concentrations being used chiefly for differential staining, rapid death and disintegration, the lower for demonstration of the gradients by differential reduction and slow disintegration. Concentrations of neutral red used were all very low but were not determined because the results differ only as regards time with all concentrations used and because the dye gradually crystallizes out of solution.

The Polar Gradients During Normal Development.

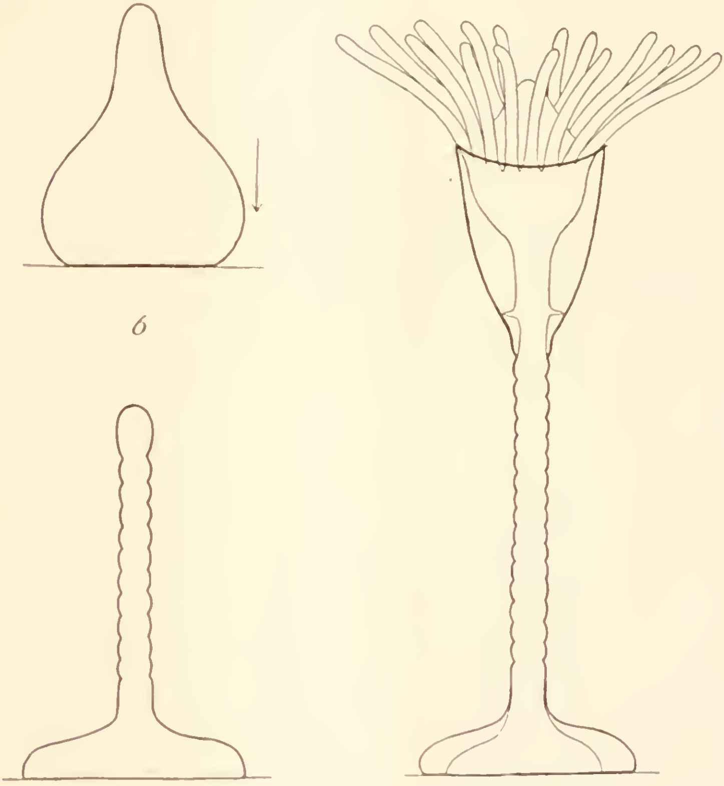
The course of early development under normal conditions is very similar in the species used as material. Cleavage gives rise to a spherical ciliated blastula (Fig. 1) which soon begins to elongate and shows polarity in the graded thinning of the cell wall decreasing from apical to basal pole, and in locomotion with the apical end in advance (Fig. 2). Immigration of cells from the basal hemisphere, chiefly from regions near the basal pole, begins within a few hours (Fig. 3) and the cells which immigrate apparently undergo further division and arrange themselves as an inner entodermal layer as elongation of the larva continues (Fig. 4). The final form of the swimming planula, attained in *Phialidium* after about forty-eight hours at laboratory temperature, is approximately that shown in Fig. 5, but a considerable amount of slow contraction and extension occurs, the same planula being at times almost worm-like in form or contracting to a form considerably shorter and broader than Fig. 5. The original apical end still precedes in locomotion, as indicated by the arrows (Figs. 2–5). After a day or more of continued swimming, either free or along the bottom, the planula attaches itself by the original



Figs. 1–5.

apical end, which shortens and thickens (Fig. 6), and the basal end begins to elongate to form the stem (Fig. 7) and this in turn gives rise at its tip to the first hydranth (Fig. 8). Stolons may arise later as outgrowths from the attached end. Stem and stolon are easily distinguished since the stem grows erect or free from the substratum and has an annulated perisarc, while the stolon grows in contact with the substratum and is without annulation. This course of development is essentially similar to that described by other investigators for various other hydroid species.

The evidence from the course of development itself, from differential cytolysis, from differential rate and amount of reduction of $KMnO_4$, from differential staining by neutral red and methylene blue and differential reduction of the latter, agrees in indicating, first the existence of a physiological gradient along the polar axis from the oöcyte to the planula stage with its high end at the apical end of egg and larva, and second, the appearance in the late planula of a second gradient at the basal end, opposite in direction to the first, and associated with the development of stem and hydranth from this end. Figs. 10 to 17, illustrating diagrammatically the polar gradients of various stages, are in part early stages of the reduction gradients with $KMnO_4$ (Figs. 10–12), in part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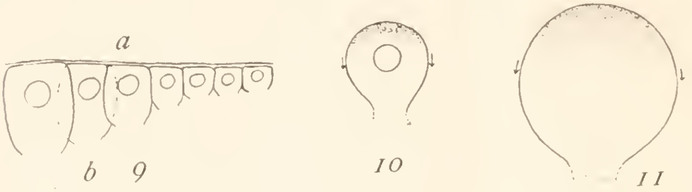
Figs. 6–8.

early stages of cytolytic gradients. Although complete series of data up to the first hydranth were obtained with cytolysis, with reduction and with differential staining, complete series of figures for the various methods are believed to be quite unnecessary since the results of all agree. The small arrows in Figs. 10–17 indicate the direction in which further progress of reduction or cytolysis occurs. As regards Figs. 10–12 it should also be noted that the shaded portions represent those regions in which the color resulting from reduction of $KMnO_4$ is deepest. All portions of the egg or later stage become diffuse yellow in permanganate within a few moments, but the differential staining of different regions becomes more and more distinct as reduction proceeds, until the increasing opacity obscures the differences and dehydration and clearing are necessary to make them visible.



FIGS. 9–11.

The growing oöcytes of *Phialidium* form a more or less regular columnar epithelium in the gonad (Fig. 9), each cell being attached by its inner end (*b*) adjoining the radial canal, while the superficial free pole (*a*) is separated from the sea water only by a very delicate membrane covering the gonad. Except in very early oöcytes, the nucleus lies nearer the free pole. Growing oöcytes of various stages, isolated by teasing, rapidly assume rounded form and the nucleus often breaks down or is extruded, but the region of attachment remains distinguishable and serves as a landmark. Such isolated oöcytes show uniformly a gradient in susceptibility with all agents used, in reduction of permanganate and in differential staining, the cytolysis or change in aggregation of the cytoplasm, the reduction and the staining beginning at the free pole and progressing toward the attached pole, as indicated in Figs. 10 and 11. Nucleated and non-nucleated egg fragments also show a gradient which is doubtless identical with that of the whole oöcyte, though the absence in most cases of any visible basis for orientation of the fragments makes complete demonstration of identity impossible. In the oöcyte of *Stomotoca* the gradient shows the same relation to free and attached poles of the cell as in *Phialidium.*

It has been repeatedly pointed out that the various lines of evidence concerning the gradients indicate very clearly that they are associated with quantitative differences in physiological condition involving metabolism as an important factor.[1] In the light of this evidence the inference is justified that the differentials in susceptibility, reduction and differential staining in these hydrozoan oocytes are indicators of a quantitative physiological gradient in the cytoplasm. The relation of this gradient to the free and attached ends of the oöcyte in the gonad suggests that it is determined by the differential exposure of the cell. The intake of oxygen and elimination of Co_2 undoubtedly occur more readily at the free end than elsewhere, and there is every reason to believe that the persistence of such a differential during the growth period will establish a physiological gradient, primarily quantitative in character, involving metabolic and physical factors. In short, the facts at hand indicate, though they do not demonstrate that the physiological polarity of the oöcyte is determined by its differential exposure in the gonad.

The gradient persists after the egg is shed, the polar bodies are formed at its high end, *i.e.*, the pole of highest susceptibility and reducing power, and the first cleavage furrow cuts through the egg from this pole (Fig. 12). During cleavage and early blastula stages a gradient is continuously present, undoubtedly the same as that of the early stages, but absence of definite landmarks makes complete demonstration of identity impossible. As soon as directed locomotion and elongation of the blastula begin, we find that the gradient is apico-basal, the high end being at the apical pole of the blastula (Fig. 13) and the planula (Fig. 14). The facts leave little room for doubt that the gradient, apparently determined by differential exposure in the oöcyte, represents the polar axis of the egg and larva. Moreover, maturation, cleavage, the differential thinning of the blastula wall and the direction of locomotion of the larva all indicate a greater physiological activity at the apical pole, the high end of the gradient, than elsewhere.

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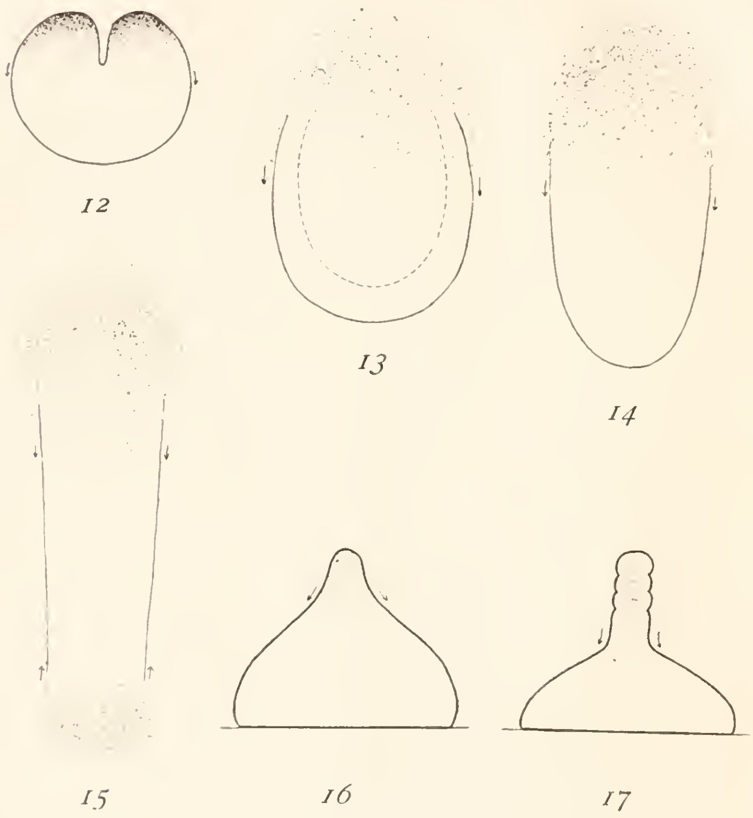
[1] For discussions of this evidence see Child, '20, '21, Chap. II.; '23b; '24, Chap. VII. and literature cited in these publications.

The question whether any gradient exists or arises in the entoderm is of interest, but the only method among those used which is available for demonstrating the presence of such a gradient is that indicating different amounts of reduction of permanganate at different levels. After the entodermal cells have formed a definite internal layer in the planula the cleared permanganate preparations usually show a slight entodermal gradient in amount of reduction, as indicated by depth of color. This gradient is similar in direction to the ectodermal gradient but the differences at differ-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Figs. 12–17.

ent levels are less than in the latter. The immigration of the entodermal cells singly and their later arrangement in a layer makes it difficult to believe that this entodermal gradient represents differences present in the cells at the time of, or preceding immigration. If it represents real differences in physiological condition, it is probably superimposed on the entoderm by its relation with the ectoderm, the more active regions of the latter determining, perhaps by transmission, greater activity in the adjoining entoderm.

As the planula elongates a second region of high susceptibility, reducing power and rate of staining gradually appears at the basal end (Fig. 15) and from this region a gradient, opposite in direction to the original gradient, develops. The stage at which this second gradient appears varies somewhat in the different species and in different cultures of the same species. Under good conditions it usually appears somewhat before the planula attains its full length. Under slightly depressing conditions it commonly appears somewhat earlier, but under strongly inhibiting conditions its appearance may be completely inhibited. The portion of the planula body over which the second gradient extends during the free swimming stage varies considerably, being usually one fifth to one third the total length. Undoubtedly the degree of development of this second gradient before attachment depends on the stage at which it first appears and on the condition of the larva.

This second gradient is the first indication of the hydranth-stem axis. As the stage of attachment approaches the activity of the original apical region decreases and the original apico-basal gradient becomes less distinct, while the new gradient at the basal end becomes longer and more distinct. During this period the swimming activity decreases and sooner or later attachment by the original apical end occurs (Fig. 16), a perisarc is secreted and elongation of the basal region to form the hydranth and stem begins (Fig. 17).

Thus far no indications of the original apico-basal gradient have been observed at the stage of Fig. 17. Apparently the only gradient present is the secondary gradient which originated at the original basal end. The objection may be raised that the presence of the perisarc and the attachment of the original apical end protect this region from the action of external agents. This, however, is not the chief factor in the low susceptibility and reducing power of this region, for when these stages are scraped from the substratum the attached surface is often left exposed, but even then the original apico-basal gradient does not appear. As a matter of fact, various experiments on susceptibility and reducing power of naked protoplasmic surfaces and those covered by perisarc have shown that, except when the perisarc is old and very thick, it is rapidly penetrated by most agents. The very thin perisarc of stages such as Figs. 16 and 17 is penetrated rapidly by $KMnO_4$ and the agents used to determine susceptibility

The tip of the growing stem which represents the high end of the secondary gradient gives rise to the first hydranth (see Fig. 8). This hydranth itself shows a marked apico-basal gradient which is a part of the hydranth-stem gradient. Each tentacle, originating as a localized region of growth on the hydranth body, represents a gradient with the high end at the tip. The tentacle gradient apparently originates in the localization of the region of growth giving rise to the tentacle. This region is not sharply defined, but its activity decreases peripherally from a central area and this decrease evidently constitutes the beginning of the tentacle gradient. The gradients of numerous other axiate organs undoubtedly arise in the same way. The localization of a region of growth giving rise to a tentacle must be determined by correlative factors in the developing hydranth. The tentacles arise at a certain level of the polar gradient and at a certain distance from each other. Each growing tentacle region, once localized, apparently dominates a certain area as does a growing plant bud and determines the course of development of this area. From this viewpoint the tentacle is a gradient in a specialized part which originates at a certain body level in reaction to the polar gradient of the body. In the species serving as material for this paper the tentacles of a circle are apparently not simultaneously determined or localized but the factors concerned in their localization are obscure.

THE PHYSIOLOGICAL SIGNIFICANCE OF THE VARIOUS METHODS.

The first question which arises is whether the axial differences in susceptibility to the various agents used result merely from differences in permeability of the limiting surfaces of the cells. That axial differences in permeability to some, if not to all agents, corresponding more or less closely to the gradients observed, do exist is probable. But that such differences are not the only, nor the fundamental factors in the gradients is indicated by various facts, some of which require brief consideration in connection with the case in hand. First, an observation made repeatedly on oöcytes of both *Phialidium* and *Stomotoca* teased from the gonad is at least suggestive in this connection. As already noted, the oöcytes are attached during the growth period and when they are teased out of the gonad rupture of the cytoplasm occurs at the attached pole and, although the injured region closes within an hour or two, the intact plasma membrane present over other parts of the surface is absent in this region for some time and granules or droplets are often given off into the water. But even while this cytoplasmic region is exposed, it does not become the high end of the susceptibility gradient or the reduction gradient. That is at the opposite pole, in spite of the fact that the agents must penetrate the intact membrane there in order to produce their effects. The only effect of the exposure of the cytoplasm at the attached pole is a little superficial cytolysis or reduction. This absence of relation between the polar gradient of the oöcyte and the injury at the attached pole is striking.

Second, as regards the action of the various agents used to demonstrate the gradients by cytolysis, it is important to note that the cytolytic gradients are the same for agents such as methylene blue and neutral red which penetrate living membranes readily and kill by accumulation within the cells and for those such as $HgCl_2$ which, in the concentrations used, alter and kill the cell surfaces rapidly and so destroy such differences in permeability as may have existed. Again, the cyanides or the CN ion apparently penetrate cells readily and produce their effects largely within the cells, according to recent views by inhibition of the catalyst of oxidation. As a matter of fact, susceptibility to KCN has been shown in various ways to be, within certain limits a very delicate indicator of physiological condition and particularly of rate of cell respiration. When susceptibility gradients are found to be essentially the same for different agents which act on living protoplasm in ways as different as KCN, $HgCl_2$ and the vital dyes in the concentrations used, it seems impossible that the differences in susceptibility can be due simply to differences in permeability. In this work on the hydroids the use of a large number of agents for lethal susceptibility was regarded as unnecessary for several reasons: first, because differential susceptibility to KCN is believed to be a valuable indication of quantitative differences in physiological condition which in most, if not in all protoplasms, are associated with oxidative processes; second, because the data obtained by different methods, lethal susceptibility, reduction and modification of development, the last to be considered in a later paper, all agree as regards the physiological gradients indicated; and third, because in the light of earlier work on many different forms, with the methods used here and with other methods, the data on hydroid development appear merely as additional evidence in agreement with that already at hand.

And finally, the physiological gradients demonstrated experimentally correspond to differences in rate of the developmental processes. The high ends of the gradients are the most active regions. Differences in permeability are in all probability concerned in the differences in activity, but if this is the case, permeability is merely one factor in the complex of metabolic and other factors which constitute physiological condition.

The basis of the general relation between physiological condition and susceptibility has been discussed elsewhere. At present it need only be pointed out that it does not depend on the particular method of action of a particular agent, but is apparently a relation between a certain degree of disturbance of any kind and the rate of change characteristic of the system disturbed. To a degree of disturbance adequate to bring about disruption or irreversible change of the system in course of time, the more active region is more susceptible because its own activity becomes a factor in producing the total or final effect (Child, '23*b*).

As regards the gradients indicated by reduction of $KMnO_4$, it has already been noted that they appear both as gradients in rate of reduction indicated by rate of appearance of the brown color and as gradients in amount of reduction indicated by depth of color when the reaction proceeds to completion in excess of the agent and the preparations are dehydrated and cleared. The possibility that a differential permeability along the axis, either of the plasma membrane of naked stages, or of the perisarc in stages possessing it, is a factor in the gradient in rate of reduction need not be denied. It is highly improbable, however, that such differences in permeability are fundamental factors in the gradients of naked stages for $KMnO_4$ in the concentrations used penetrates rapidly and even alters the visible aggregation of the protoplasm before the reduction gradient becomes distinct. Any differences in permeability which may have been present must be destroyed early in its action. Moreover, the possibility that differential permeability determines the gradient in amount of reduction as indicated by depth of color due to oxides precipitated throughout the cell or cells, seems to be eliminated. And finally, the fact that the gradients in rate and in amount of reduction are the same for a particular stage and are also identical with the gradients in susceptibility indicates clearly enough that the different methods of demonstrating the gradients are merely different ways of making evident one aspect or another of the general quantitative gradation in condition of the living protoplasmic system.

That the gradients indicated by reduction of $KMnO_4$ are closely associated with the processes of living in the protoplasm is further shown by the fact that individuals of various stages killed in various ways and then placed in permanganate reduce much less of the agent than living individuals and show, either no traces of axial gradients, or in some cases slight traces for a short time after the action of the killing agent. For example, when developmental stages are placed in alcohol, $HgCl_2$ or various other agents used for histological purposes, then washed and brought into $KMnO_4$ after various periods in the killing agent, it is found that a few seconds in most agents is sufficient to obliterate in large measure the differences on which the reduction gradient depends, though slight traces of a gradient may persist in some cases, even for several hours. In material carried up to 80 per cent. alcohol, returned to water after a day or two and then brought into permanganate no traces of the reduction gradient have thus far been found. The persistence of traces of the gradients for a short time after action of a fixing agent can scarcely be due to the persistence of a permeability gradient, even if such were present in the living animal. Another interpretation appears more probable, viz., that complete inactivation of the oxidizing enzymes does not always occur at once in the fixing agents, consequently traces of the original differential may persist for a short time. Individuals killed before exposure to permanganate never become opaque black but remain yellow or brownish indefinitely in excess of the agent. Evidently killing before exposure decreases greatly the reducing power of the protoplasm.

The gradients of differential staining with neutral red and the higher concentrations of methylene blue are visible only during the early stages of staining in living material. As the staining of living material progresses the regional differences in depth of color gradually disappear and, so far as the eye can distinguish, the staining becomes uniform throughout long before the depth of color is sufficient to obscure differences. But if staining is continued until cytolysis occurs the cytolytic gradients are the same for any particular developmental stages as those observed with other agents.

The differential staining gradients may represent primarily the differences in permeability of different regions to the dyes, but if appearances can be trusted these differences in permeability disappear as the toxic action of the dyes progresses and in spite of the fact that all regions seem to be stained uniformly, the regions which are more susceptible to other agents and which show greater reducing power are more susceptible to these dyes. Here as in other forms, such differential permeability as may exist appears to be merely one aspect of the differences in physiological condition which constitute the gradients.

In the case of methylene blue the ability of the living protoplasm to reduce and decolorize the dye rather rapidly introduces another factor and makes possible demonstration of the gradients as gradients in rate of reduction and decoloration, particularly in the blastula, planula and later stages, in which the rate of metabolism is higher than in early stages. The reduction gradients with methylene blue appear only in relatively low concentrations or after removal to water before staining has progressed too far, but the concentrations and periods of staining suitable for this purpose differ with the different species. For example, the planulæ of *Phialidium* show differential staining with the high regions of the gradient or gradients most deeply stained in the early stages of staining with methylene blue 1/1000, but in 1/5000 scarcely a trace of differential staining occurs, the whole larva staining more or less uniformly. In 1/20,000 there is no appreciable staining during the first two hours of exposure, but after six hours many planulæ show a reversed staining gradient. In the shorter planulæ the depth of staining is least at the apical end of the larva and increases basipetally; in the longer planulæ, in which the second gradient is already present, both ends are slightly or not at all stained and the depth of color increases toward the middle region. Similar reversal of the staining gradient has been observed with low concentrations of methylene blue in various multicellular forms and in ciliate infusoria, including *Paramecium*. These reversed staining gradients in low concentrations result from reduction of the dye to the leuco-compound in the protoplasm. Below a certain concentration, which must be determined experimentally for each species, the higher levels of a gradient reduce the dye as rapidly, or almost as rapidly as it enters and more rapidly than lower levels. Consequently the depth of staining is least at the high end and increases toward the low end of a gradient. Even with concentrations high enough to stain the high regions of the gradients more deeply the reduction gradients often appear when the animals are returned to water before the staining has progressed far. Apparently the reduced dye is toxic, for death and cytolysis finally occur in concentrations so low that the high regions of the gradients which disintegrate first are only slightly or not at all stained. It is improbable that the toxicity of such concentrations, *e.g.*, 1/20,000, can be due to impurities.

CONCLUSION.

The evidence from the study of the gradients during development agrees with the data of observation in showing, first that the polarity of the oöcyte becomes the polarity of the early planula, and second that a complete reversal of polarity occurs in the course of transformation of planula into hydroid. A simple interpretation of this reversal is possible in terms of physiological gradients. The hydranth-stem axis apparently arises as a bud from the original basal end of the planula. The appearance of the second gradient at the basal end as the planula elongates suggests that the new axis arises through physiological isolation of this basal region from the dominant apical region in consequence of increase in length. Decrease in activity of the apical region, which is indicated by the lower susceptibility of these, as compared with earlier stages of the planula, may also decrease the range of dominance and so play a part in the physiological isolation of the basal region. In these larval stages the range of dominance is undoubtedly very short because the mechanisms of transmission are rudimentary (Child, '15, pp. 149–151) and physiological isolation may occur at a very short distance from the dominant region. In consequence of the isolation the cells at the basal end become more active and lose whatever differentiation they have attained, while the activity of other parts continues to decrease with the progress of differentiation, until the region originally basal and least active becomes the most active region of the body. With further development and with the differentiation of a rudimentary nervous system the range of dominance increases, as in the development of other forms, and the hydranth which arises at the high end of the secondary gradient comes to dominate the whole body, until further increase in length leads sooner or later to physiological isolation again and budding begins. The reversal in direction of polarity in the hydranth-stem axis apparently results from the fact that in the course of elongation of the planula the extreme basal region is the first part to be isolated and to become more active. This precedence makes it the high end of the new gradient. It is evident from the work of many investigators that polarity in the hydroids is extremely labile and readily altered by external conditions. Every hydroid bud represents a new gradient and, so far as can be determined, this bud which forms the first hydranth differs from those which later give rise to branches only in its position at the opposite end of the polar axis from the original dominant region. In that respect it is similar to the "axial heteromorphoses" which are characteristic features of the normal budding and of the reconstitution of stem pieces in *Tubularia* and many other hydroids (Child, '07*a, b* and literature there cited; '15, pp. 91, 92, 133–37). The term "heteromorphosis" is merely descriptive and leaves us without any interpretation of the phenomena concerned. So far as analyzed physiologically, all cases of so-called axial heteromorphosis are essentially cases of budding which involve the origin of a new polar gradient. Actually every hydroid bud and every bud in a multiaxial plant is as truly a heteromorphosis as are these cases of buds arising at the basal end of an axis. All such buds represent the determination of new physiological axes and the conditions, external and internal, determine in each case where the new axes shall appear.

In some of the tubularian hydroids, *e.g.*, *Corymorpha* (Torrey '07, and my own observations), the hydranth-stem axis arises from the original apical end of the planula and although the secondary gradient appears sooner or later at the basal end, physiological isolation is not complete and the secondary gradient gives rise, at least in its earlier stages only to stolon axes, which are inhibited axes (Child, '23*a*). In these forms, then, the primary gradient persists as the gradient of the hydranth-stem axis.

Summary.

1. The axial gradient and its changes during development were demonstrated in *Phialidium* from oöcyte to hydroid stage by differential susceptibility to various agents, differential reduction of $KMnO_4$ and methylene blue and differential vital staining. Developmental stages of *Gonothyræa* and *Stomotoca* served as comparative material. The results of the different methods agree among themselves and with the observed facts of development.

2. In *Phialidium* and *Stomotoca* the polarity of the oöcyte is indicated by a gradient with high end at the free pole, low end at the attached pole. This gradient is directly related as regards direction to the differential exposure of the oöcyte in the gonad and it is suggested that the polarity of the oöcyte is determined by this differential exposure.

3. The high end of the oöcyte gradient becomes the apical pole of the egg at maturation and in early cleavage stages. During later cleavage a gradient is continuously present, but absence of landmarks makes complete demonstration of its identity with the gradient of earlier stages impossible, though there is no reason to doubt that it is the same. From the beginning of locomotion on the identity of the gradient is certain, the high end being the apical, the low end, the basal pole of the larva.

4. As the planula elongates a second gradient opposite in direction to the primary gradient appears at the basal end of the larva and gradually extends toward the apical end. Soon after this gradient appears the planula of *Phialidium* and of *Gonothyræa* attaches itself by the original apical end and the second gradient becomes the hydranth-stem axis. Attachment of the planula of *Stomotoca* was not observed.

5. The origin of this second polarity, opposite in direction to the first, is interpreted as a result of physiological isolation of the basal end of the planula in consequence of increase in length. From this viewpoint the hydranth-stem axis represents a process of budding not essentially different physiologically from other processes of budding in hydroids and various other forms.

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STUDIES OF COAT-COLOR AND FOOT PIGMENTATION IN SUBSPECIFIC HYBRIDS OF *PEROMYSCUS EREMICUS*

F. B. SUMNER AND R. R. HUESTIS.

I. Introduction.

In several previous papers[1] the senior author has reported upon inheritance, variation, correlation and allied phenomena in crosses between several subspecies of *Peromyscus maniculatus*. A considerable number of measurable characters were dealt with in the course of these studies, some of these relating to the absolute or proportional size of various bodily parts, others to the pigmentation of the hair and skin.

It was early evident not only that the characters distinguishing two subspecies, taken collectively, did not depend upon single mendelian allelomorphs, but that the same was true of each of the various subspecific differences, taken separately. So far as studied, all of these characters followed the "blending" type of inheritance. If they were dependent upon mendelian factors at all, several or many of these were concerned in the production of each character difference. Moreover, studies of correlation showed that in many cases these separate characters were inherited independently of one another.

About three years ago, the junior author began the study of another species of *Peromyscus*, *P. eremicus*, a form differing sufficiently from *P. maniculatus* to be assigned to another subgenus.[2] Two subspecies were dealt with, *P. eremicus eremicus* and *P. e. fraterculus*. The parent stock of the former race was trapped in Death Valley, California, in 1920, the latter animals were taken in the neighborhood of La Jolla. In appearance, these two geographic races differ in the same manner as do desert and coast-dwelling representatives of various other species of rodents. The difference is about as great, for example, as that between *P. maniculatus sonoriensis* and *P. maniculatus gambeli.*

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[1] *Journal of Experimental Zoölogy*, Oct., 1923, and papers therein cited.

[2] For a brief summary of the results of these studies see Huestis, *Proc. Nat. Acad. of Sci.*, 1923. A fuller account is now in press.

The material comprised in these studies included 208 specimens of the parent races, *eremicus* and *fraterculus,* 199 F_1 and 147 F_2 hybrids. No bodily measurements were made of these mice, nor were skeletons saved. Skins were, however, prepared according to the method long practiced by the senior author.[1] Later, the feet were removed from the dried skins for a study of the pigmentation of the soles. After removal, they were first soaked for a day in distilled water, next transferred to 70 per cent. alcohol, then to equal parts of alcohol and glycerine, and finally to pure glycerine. After this treatment, the feet were graded according to a scale previously adopted by the senior author.

The series of skins upon which these studies were made were superior in two respects to those earlier employed by us in the study of *Peromyscus.* In the first place, the numbers were larger, both for the pure races and the hybrids. Secondly, the animals were skinned at a somewhat greater age, eight months being adopted as a minimum instead of 6½ months, as had been the previous practice.[2] Thus the hair of these mice seldom showed any traces of the second molt, having passed into the third (first "adult") pelage.[3]

From these prepared pelts small samples of skin, with the accompanying hair, were removed with a sharpened cannula, having a diameter of about 1.5 millimeters. The hairs were subjected by the junior author to quantitative determinations of two sorts. (1) They were grouped, on the basis of their size and pigmentation, into four somewhat arbitrarily distinguished classes, and the number of each class comprised in the sample was counted. (2) The length of the subterminal ("agouti") band and of the terminal region, distal to this, were measured for ten of the "class A" hairs in each sample. Likewise, the total length of the longest hairs of the sample, *i.e.*, the maximum hair length of this region, was determined in each case.

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[1] Sumner, *Journ. Exp. Zoöl.,* Aug., 1909 (p. 101); described more fully by Collins, *Journ. Exper. Zoöl.,* Aug., 1923 (p. 48).

[2] A few which died at an age less than 8 months were included, provided that the hair of these showed no trace of molting. On the other hand, some others were several months older than this minimum age, owing to their having been kept for breeding purposes.

[3] At the age adopted for the earlier series (6½ to 7 months) a certain proportion of animals had not fully completed the second molt.

It was already known that the general coat color of the individual depended upon the proportional number of different types of hairs in the pelage, as well as upon the relative length of the subterminal yellow band in the "agouti" hairs and some other characters. If, therefore, the color differences between these various geographic races of mice were due to differing combinations of mendelian unit-factors, such factors would be more likely to reveal themselves in the hereditary behavior of these more elementary characters than in that of the mass effects, as shown by photometer readings of considerable areas of the pelage. To what degree, if at all, this theoretical expectation has been realized, will be discussed later.

In the following pages, we shall deal with these mass effects first, before proceeding to a consideration of the various microscopic hair characters which form their basis. This seems the more logical order to follow, despite the fact that in the case of this particular series of skins the microscopic studies preceded the photometric ones.

II. General Coat Color as Determined by the Tint Photometer.

The Hess-Ives Tint Photometer and its use in the study of mammalian pelages have already been discussed briefly by the senior author in several previous papers.[1] Pending the preparation of a more adequate account of this procedure, a very brief restatement of the technique employed must suffice.

A rectangular area of the pelage, together with an equal area of a "standard" white, are viewed in juxtaposition through three successive color screens (red, green and blue-violet). In each case, the light from the standard white is cut down by a shutter until the luminosity (and necessarily the color as well) is equal to that of the object under examination. The readings of the lever controlling this shutter, which are expressed in hundredths, furnish the data from which the color and luminosity values here employed are computed.

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[1] For most complete statements see Sumner and Collins, *Journ. Exp. Zoöl.*, Oct., 1922, pp. 289, 294.

The values employed in the studies thus far made are the percentages of "black," "white" and "color" in the sample. For reasons which we shall not justify here, the difference between the highest reading (always red, in our material) and 100 is regarded as the percentage of "black"; the lowest reading (blue-violet, in our material) is regarded as being likewise the percentage of "white"; while the balance (difference between the sum of these two and 100) is regarded as the percentage of "color." By "color" is here meant the free color, *i.e.,* that which remains of the two higher readings after deducting the amount regarded as combining to form white light. The spectral position of this "color" is not, of course, given in any direct way by these readings. It reaches its maximum, in all cases, somewhere between red and yellow, its nature being indicated crudely by the ratio between "free" red and green (R : G ratio). This ratio is high in reddish skins, low in yellowish ones.[1]

In the present studies, two dorsally situated and symmetrical areas of the pelage were selected, one being in the shoulder region, the other a little anterior to the base of the tail. The means of the values for the two areas were employed in the computations, and each value was likewise based upon two readings, made upon different days.

In Table I. are given the means and standard deviations for each of these "characters," the two parent races and the two hybrid generations being considered separately. The number of individuals here given is in each case smaller here than that employed by Huestis for the study of microscopic hair characters. This is because certain pelts which could be used for the latter purpose were not suitable for the former, owing to injury or to molting within the areas under examination.

The salient facts shown in the foregoing table are as follows:

1. As was evident from a casual inspection of skins, *P. e. fraterculus* is a considerably darker race than *P. e. eremicus.* This is indicated by a higher percentage of "black" in the former and by the lower percentages of "white" and "color."

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[1] To illustrate: suppose that the readings for red, green and blue-violet are 25, 15 and 11, respectively We have in this case, 75, 11 and 14 respectively, as the percentages of black, white and color. The R:G ratio is 3.5.

TABLE I.

	Number	Black.		White.		Color.		R : G Ratio.	
		Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.
eremicus	92	77.56 ± .14	1.97 ± .10	13.41 ± .07	1.04 ± .05	9.03 ± .09	1.29 ± .06	2.49 ± .013	.18 ± .009
fraterculus	93	84.02 ± .09	1.36 ± .07	9.07 ± .06	.80 ± .04	6.31 ± .07	.99 ± .05	2.59 ± .014	.21 ± .010
F$_1$ hybrids	177	79.56 ± .08	1.50 ± .05	12.32 ± .04	.88 ± .03	8.12 ± .05	1.04 ± .04	2.56 ± .009	.18 ± .006
F$_2$ hybrids	132	80.44 ± .12	2.01 ± .08	12.22 ± .06	1.07 ± .04	7.34 ± .07	1.27 ± .05	2.44 ± .010	.17 ± .007

2. The R:G ratio is slightly, though perhaps significantly, higher in *fraterculus* than in *eremicus*. In other words, the darker race seems to be relatively redder, the paler race relatively yellower. It was earlier observed that a dark race (*rubidus*) and a pale race (*sonoriensis*) of another species (*maniculatus*) differed in this same direction, though to a greater extent.

3. The mean values for the F_1 generation of hybrids lie, in each case, between the values for the two parent races, though they are not in any case midway in position.

4. The mean values for the F_2 generation differ somewhat from the F_1 values, but they are none the less intermediate between those of the parent races with the exception of the figure for the R:G ratio.

5. The standard deviations for the F_2 generation exceed those for the F_1 in the case of black, white and color, the differences being 5.4, 3.8 and 3.6 times their probable errors, respectively. There is no such increase of variability, however, in the case of the R:G ratio, the standard deviations for which are approximately equal in the two generations. It will be noted that the three former " characters " differ rather widely in the two parent races, whereas the difference in the latter case is slight.

The statistical certainty of the color differences between the subspecies which were crossed, and the fact that these differences persist, apparently undiminished, after several generations in captivity, show that they are largely genetic in their nature. The same is not so evident with respect to the individual differences within a single race or within a group of hybrids of similar origin. Observation shows that part of these differences are due to age or to condition of molt; while season is perhaps likewise responsible to a certain extent. Previous studies[1] have shown, however, that a certain fraction of this individual variability has a genetic basis. For the present material, coefficients have been computed, indicating the degree of parent-offspring correlation with respect to the four values employed in our computations (black, white, color and the R:G ratio). The resulting figures are given in the following table.

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[1] Sumner, *Journ. Exp. Zoöl.*, Oct., 1923.

Table II.

Parents and Offspring Correlated.	Number of Parents.	Number of Offspring.	Black.	White.	Color.	Mean of Preceding Three.	R : G Ratio.
eremicus—F_1 .	33	153	+.146 ±.068	+.197 ±.067	+.283 ±.064	+.209	−.047
fraterculus—F_1	18	176	+.321 ±.062	+.220 ±.065	+.331 ±.061	+.291	+.114
F_1♂—F_2	30	132	+.274 ±.069	+.272 ±.069	+.216 ±.071	+.254	−.002
F_1♀—F_2	40	126	+.188 ±.071	+.210 ±.071	+.199 ±.071	+.199	+.094

These figures show that part of the individual variability in coat color is genetic. The correlation is, however, rather low, the mean of the 12 figures for "black," "white" and "color" being only 0.28. This indicates that a large proportion of the variability is due to non-genetic causes.

Furthermore, these and previous data make it doubtful whether individual differences in the R:G ratio are inherited at all, despite the fact that two races may differ rather widely from one another in respect to this character.

III. Microscopic Hair Characters.

The junior author has already published a brief account of his findings with respect to certain hair characters which he has been able to determine quantitatively with the microscope.[1]

Table III. gives the mean values as well as the variability of seven "characters" in the two parent races and in the F_1 and F_2 generations of hybrids. It is evident that the four mean values comprised in the first half of this table are not independently variable, since their sum is in every case 100. The classification of hair types here included is somewhat arbitrary, as the various classes intergrade completely with one another. The hairs of these animals may be distinguished as banded (classes *A*, *B*, *C*) or as non-banded (*D*). In the latter very dark ("sepia") pigment is present throughout the entire length of the hair. In the former, this dark pigment is interrupted, toward the distal end, by a yellow cross band of varying extent, giving rise to the condition known to geneticists as "agouti."

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[1] *Proc. Nat. Acad. Sci.*, Oct., 1923.

Table III.

	Number.	Percentage *A* Hairs.		Percentage *B* Hairs.		Percentage *C* Hairs.		Percentage *D* Hairs.	
		Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.
eremicus..........	108	50.39 ± .34	5.23 ± .24	29.43 ± .31	4.72 ± .22	8.39 ± .22	3.37 ± .15	11.52 ± .20	3.01 ± .14
fraterculus.........	100	59.27 ± .30	4.44 ± .21	18.32 ± .22	3.23 ± .15	9.82 ± .16	2.41 ± .11	12.27 ± .21	3.13 ± .15
F$_1$ hybrids........	199	56.42 ± .23	4.73 ± .16	23.37 ± .18	3.71 ± .12	9.15 ± .13	2.74 ± .09	10.71 ± .16	3.43 ± .12
F$_2$ hybrids........	147	56.25 ± .31	5.48 ± .22	22.95 ± .21	3.76 ± .15	9.04 ± .17	3.07 ± .12	11.52 ± .21	3.80 ± .15

Table III—*Continued*.

	Total Length of Hair in Mm.		Length of Agouti Band in Tenths of a Mm.		Length of Distal Tip in Tenths of a Mm.	
	Mean.	St. Dev.	Mean.	St. Dev.	Mean.	St. Dev.
eremicus..........	12.40 ± .06	.87 ± .04	14.21 ± .09	1.41 ± .06	3.35 ± .02	.39 ± .02
fraterculus.........	11.08 ± .04	.61 ± .03	8.38 ± .06	.93 ± .04	4.36 ± .03	.38 ± .02
F$_1$ hybrids........	11.23 ± .03	.67 ± .02	12.06 ± .06	1.29 ± .04	3.78 ± .02	.38 ± .01
F$_2$ hybrids........	11.48 ± .04	.79 ± .03	11.32 ± .10	1.87 ± .07	3.90 ± .03	.50 ± .02

Of the banded hairs, class *A* includes those which are slender throughout (they are also relatively short), while class *B* includes those which broaden out through the region of the subterminal band. Class *C* comprises hairs which are intermediate between *B* and *D*, and for which the creation of a separate class has seemed justifiable.

In the lower section of Table III. will be found the "total length," together with the lengths of the two distal subdivisions of the hair, all of these values being based upon micrometer measurements. The first of these figures represents the length of the longest hairs in each sample, the two last are means derived from the measurement of ten "*A*" hairs in each sample.

It will be noted that the proportion of *D* (all-black) hairs is slightly greater in the darker race (*fraterculus*) than in the paler race (*eremicus*), but that the chief difference between the two lies in the greater proportion of *B* (broad "agouti") hairs in *eremicus*. This is doubtless partly responsible for the paler and more highly colored pelage of the latter subspecies. As regards the slender, banded hairs of the *A* class, the relations are reversed, the darker race (*fraterculus*) having proportionately more than the paler race. Owing to the relative shortness and slight width of these hairs, they contribute, however, much less to general color effect of the pelage.

Eremicus appears to have slightly longer hairs than *fraterculus*, but the fact of chief interest revealed by the linear measurements is the considerably greater mean length of the "agouti" band in this race. The figures here given are based upon hairs of the "*A*" class, but similar relations would have held in the case of the "*B*" hairs,[1] and this difference in the length of the yellow subterminal band is without doubt another of the important factors which are responsible for the color differences between these two races.

Further scrutiny of the table reveals the fact that in the case of six out of seven of these "characters," the mean values of both hybrid generations are intermediate between the values for the parent races. The single exception relates to the percentage of *D* hairs, a character in respect to which the parent races differ but slightly.

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[1] It was less practicable to employ these in the present measurements.

From the standpoint of current genetic theories, the figures of chief interest in this table are those relating to the relative variability of the F_1 and F_2 generations of hybrids. It will be noted that in all of the seven cases under comparison the standard deviation is larger for the second generation than for the first, although in some cases the difference is not statistically significant. The probability quotient (difference ÷ probable error) ranges from 0.3 to 7.2, the mean of the seven figures being 3.3. It is to be remarked that the increase of variability is most certain ($D/E = 7.2$) in respect to one of the characters (agouti band) which differ most widely in the two parent races. But it must likewise be noted that the increase is quite non-significant ($D/E = 0.3$) in the case of the other most distinctive racial difference (percentage of *B* hairs.)

It is of interest to compare the evidence for segregation (so far as this may be inferred from the F_2 variability), as shown by this series of determinations and by those made with the tint photometer. Omitting the figure for total length of hair, which has little to do with pelage color, we have the following $\frac{D}{E}$ quotients for the "characters" thus far considered:

B hairs	0.3	White	3.8
D "	1.9	Black	5.4
C "	2.2	Tip	5.5
A "	2.8	Agouti	7.2
Color	3.6		

Here it will be seen that the mean of the quotients for the four classes of hairs is 1.8, that for black, white and color being 4.3, and that for the two measured segments of the individual hairs being 6.3. So far as may be judged from the foregoing figures, it can hardly be said that these microscopic hair characters show, on the whole, any greater evidence of segregation than do the color values for the aggregate pelage. The figure for black is exceeded (significantly) by only one other figure, that for the agouti band, while the figures based upon the hair counts are the lowest of the series. If we accept the degree of segregation[1] as a safe index of the number of genetic factors underlying a given character, we must conclude that only one of the foregoing characters (agouti band) is more elementary, genetically speaking, than the crude value for "black" in the pelage. While the data at hand do not warrant any such simple inference, we may safely affirm that microscopic analysis has thus far helped us little in the search for unit factors underlying the coat color of *Peromyscus*.[2]

That we are here dealing in part with genetic differences is plain from a consideration of parent-offspring and fraternal correlations based upon these characters. The following figures are the means for six of the characters, the coefficients for "tip" not having been computed:

Parent-offspring

Eremicus — F_1	+ .302
Fraterculus — F_1	+ .267
F_1 ♂ — F_2	+ .254
F_1 ♀ — F_2	+ .205

The mean of these figures is 0.257, being thus somewhat greater than the mean of the figures (0.238) for black, white and color. Correlation is not equally strong for all of these characters. The mean of the figures for the *A*, *B*, *C*, and *D*, hairs is 0.227, that for total length being 0.314, and that for the agouti band 0.319. These figures suggest that the proportion of non-genetic variability is considerably greater in the case of the hair counts than in the case of the linear measurements, a fact of possible significance in connection with the inferior evidence for segregation shown by the former characters.

It is to be noted that the mean correlation between the pure-race parents and their F_1 offspring (0.284) is somewhat greater than that between the F_1 parents and their F_2 offspring (0.229).[1] This condition is paralleled by that shown in Table II. for black, white and color. It might be explained as due to the segregation of factors in the F_2 generation, a process which would obviously weaken the correlation between members of this generation and their parents. In this connection it is also worth noting that the coefficient of fraternal correlation is higher for the F_1 generation (.356) than for the F_2 generation (.328), a fact which is in accord with the same interpretation.[2]

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[1] Strictly speaking, these quotients indicate the probability of the existence of significant differences, rather than the amount of such differences. With equal numbers and equal variability, however, they are proportional to the magnitude of the differences.

[2] A circumstance tending to diminish the validity of these comparisons is the varying degree of accuracy of the technique upon which the measurement of the various characters rests. The greater the observational error, the greater, of course, will be the proportion of the total variability which is due to non-genetic causes. Concerning the relative magnitude of this factor in the different cases, we have little basis for opinion.

IV. Foot Pigmentation.

The feet, prepared as above indicated, were graded according to the depth of pigmentation in the skin of the soles. In the present series, both feet of each specimen were independently graded, the mean of the two recorded values (in the exceptional cases where they differed) being employed. The following figures give the means and standard deviations for the parent races and the two generations of hybrids, the numbers being approximately the same as in the case of the skins:

	Means.	St. Dev.
Eremicus	0.45 ± .03	0.48 ± .022
Fraterculus	2.02 ± .05	0.79 ± .038
F_1 hybrids	1.65 ± .03	0.60 ± .020
F_2 hybrids	1.72 ± .04	0.71 ± .028

The three salient facts shown by the foregoing figures are (1) the much heavier pigmentation of the feet of *P. e. fraterculus*, as compared with *eremicus*, the mean difference being somewhat greater than that between *P. maniculatus gambeli* and *P. m. sonoriensis* from the same two localities; (2) the intermediate position of both hybrid genertions, which, however, average darker than the mean of the two parental figures and (3) the greater variability of the F_2 generation as compared with the F_1. We thus have much the same situation here as in the case of the other characters which have been discussed.

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[1] It should be added, however, that this relation holds for only 3 of the 6 "characters," taken singly.

[2] The circumstance that the F_2 broods resulted, to a considerable extent from the mating of sibs would tend to increase the second of these figures, and thus to diminish the difference between the two hybrid generations in respect to fraternal correlation.

In connection with certain bone measurements, the authors showed that a higher degree of bilateral asymmetry occurred in the F_2 generation of subspecific hybrids than in the F_1 generation.[1] We have accordingly compared the two hybrid generations of this cross in respect to asymmetry in the pigmentation of the feet. The number of cases in which the right and left feet were assigned to different grades was, however, very small. Four such cases occurred among the 197 F_1 individuals and five among the 147 F_2 individuals. The larger proportion among the latter cannot be regarded as significant.[2]

The following coefficients were computed, expressing the degree of correlation between parents and offspring in respect to foot pigmentation:

Eremicus — F_1	+ .097
Fraterculus — F_1	+ .150
F_1 fathers — F_2	+ .342
F_1 mothers — F_2	+ .273

The low degree of correlation between the pure race parents and their F_1 offspring (mean .128) is believed to be due to the wide range in the age of the parent stock, in comparison with the hybrid animals, which were almost homogenous in this respect. Independent data have shown us that young animals have darker feet, and that the depth of pigmentation decreases with age, at least up to a certain point. Variability due to this cause would of course weaken correlation.

Fraternal correlations for this character are .358 ± .043 and .198 ± .054, for the F_1 and F_2 generations respectively. The difference here is only 2.3 times its probable error.

V. INTRA-RACIAL CORRELATIONS.

A considerable number of coefficients of correlation have been computed in order to ascertain to what degree certain characters tend to vary together among the individuals of a single race. Most of these need not concern us in the present paper. Some of them are of little biological interest, and some, indeed, express quantitative relations which are merely due to mathematical necessity.

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[1] *Genetics*, Sept., 1921.

[2] The negative results in the present case may well be due to the relative crudity of the scale employed. The deviations from symmetry recorded for the paired bones were only detected by exact measurements.

The point of chief interest, perhaps, is to learn whether two characters, both of which differ in the two races here dealt with, tend to be correlated within the single race. Thus, among other differences, the subspecies *eremicus* is paler and more highly colored than *fraterculus*, has more hairs belonging to the "*B*" class, and less belonging to the "*D*" class, has somewhat longer pelage, a longer subterminal band in the agouti hairs, and a shorter dark segment distal to this, and considerably less pigment in the soles of the feet. To what extent are these character differences associated together within the single race?

Attention has already been called to the fact that in various subspecies of *Peromyscus maniculatus*, as well as in hybrids between these, there seems to be little or no correlation between the darkness of the pelage and the depth of pigmentation of the feet.[1] This is true despite the existence of a marked tendency for darker *races* to have darker feet.

The present studies bear out this conclusion. Separate coefficients have been computed for the males and females of the *eremicus* and *fraterculus* series, as well as for the F_1 and F_2 hybrids. Of these eight coefficients, five are positive, two negative and one 0. The figures range from — .067 to + .253, the weighted mean of the series being + .056. The only figures in the series which are of possible significance are those for *fraterculus*. In any case, we believe that the slight correlation sometimes shown between these two characters is due to heterogeneity in age. Younger mice (even when the pelage is "adult") tend to have slightly darker pelages and slightly darker feet, a circumstance which would insure a certain amount of correlation in a series of mixed age.

In some other cases, however, characters which are associated together as racial attributes have been found to be correlated within the individuls of a race. Thus, positive correlations were found in each of the four groups[2] between the maximum length of the hair and the percentage of *B* hairs (mean = + 1.68), as well as between the length of the agouti band and the percentage of *B* hairs (mean = + .240), while negative correlations were found between length of hair and percentage of *D* hairs (— .187) and between length of agouti band and percentage of *D* hairs (— .221). Such relations suggest the possibility that certain elements of the subspecific complex of characters depend upon factors which are closely linked, or that they may have, in part, a common factorial basis. That this is not true of all the characters distinctive of a subspecies is shown by the case of "black" and foot pigmentation, above referred to, and by various other facts derived from a study of the present and of previous series of animals.

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[1] Sumner, *American Naturalist*, May–June, 1923.

[2] *Eremicus, fraterculus*, F_1 and F_2 hybrids. Here the sexes have been combined in the case of each group.

Where correlation between two characters has appeared, whether positive or negative, there appears to be a tendency for this correlation to be greater in the F_2 series of hybrids than in the F_1. While such a condition has not been invariable, the tendency seems to be a real one and not due to accident.[1] Several of these differences taken singly, are of considerable statistical probablity. This phenomenon is, of course, in harmony with the view that the increased variability of the F_2 generation results, in part at least, from mendelian segregation. Whether the correlation of two characters be due to their having some common factorial basis, or to their being dependent on linked factors, the result of segregation would be to increase the correlation between them.

VI. Conclusions.

In a previous paper[2] the authors have discussed the behavior in heredity of small deviations from bilateral symmetry among paired bones. The two outstanding results of that study were (1) that such departures from bilateral symmetry were not inherited, but that (2) we none the less met with the customary increase in the variability of these characters in the F_2 generation of a subspecific cross. From these facts, the inference seemed reasonable that the increased variability of the second hybrid generation was not in itself trustworthy evidence of mendelian segregation.

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[1] In a previous study of *Peromyscus* hybrids (*Journ. Exp. Zoölogy*, Oct., 1923), little evidence was found for such an increase in correlation in the second hybrid generation. Several other correlations which were computed subsequently have rendered such an increase more probable, even for these earlier series.

[2] Genetics, September, 1921.

From a study of three series of hybrids, derived from two different subspecific crosses, the senior author was later confirmed in the belief that there was little if any proportionality between the increased " spread " of the F_2 generation and the degree of difference between the parent races in respect to the character in question. Cases were pointed out in which a considerable difference between the parent races was associated with little or no increase in hybrid variability, while in other cases, in which the parents did not appreciably differ, the appearance of " segregation " was very marked.

For the present series of hybrids and the small number of characters here dealt with the picture is decidedly different. There is a considerable degree of proportionality between the amount of difference between the parent races and the increased variability of the F_2 generation. In order to test this point more precisely, a correlation table (which it is not necessary to reproduce here) was drawn up. The " characters " under consideration were the twelve which have been dealt with in the present paper. In one column were placed, not the absolute differences between the means of the parental races, in respect to the various characters, but the differences expressed in terms of the variability of these characters.[1] In the other column were placed the differences between the standard deviations of the F_1 and F_2 generations, taken as percentages of increase.[2] Thus the vast differences in the magnitude of the original values (due to differences in the units employed) were largely eliminated.

A graphic presentation of these data revealed the existence of a considerable degree of proportionality between the two series of figures, the only serious discrepancy relating to the behavior of the "*B*" hairs (see above). The extent of the correlation between the two series is indicated by a coefficient of $+ .73$. We readily admit, however, the inconclusiveness of a correlation coefficient based upon 12 pairs of variables, particularly since the characters in queston are not wholly independent of one another, but are themselves, in some instances, rather strongly correlated. On the other hand, it hardly seems likely that this entire correspondence is accidental.[1]

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[1] Each difference between the parental means was divided by the mean of the parental standard deviations for the character in question.

[2] *I.e.* each difference was divided by the mean of these two standard deviations

According to the theory of "multiple factors," we should, in general, expect the increase of hybrid variability to be greater, in proportion as the parental difference was greater. Consideration of the simplest possible case, involving a single pair of factors, shows this to be true. Thus, the positive correlations here reported—granting their significance—are in harmony with this interpretation.

To sum up, then, the results of our present inquiries: (1) We find, for a number of measurable characters, affecting the pigmentation of the hair and skin, a blending type of inheritance, both in the F_1 and F_2 generations, following a subspecific cross.

(2) The variability of these characters is, with a single exception, somewhat greater in the second hybrid generation than in the first.

(3) This increase in variability is greatest for the length of the subterminal yellow band, least of all (slightly negative) for the "red:green ratio." The former character is the one which differs most widely in the two parent races, and is likewise most strongly hereditary, as judged by parent-offspring correlations. The latter differs but slightly in the two parent races, while the individual differences within a race do not appear to be inherited at all. With one conspicuous exception, there is a rough proportionality between the amount of the difference between the parent races, in respect to a given character, and the amount of the increase of variability in the F_2 generation.

(4) In general, the coefficients of correlation between F_1 parents and their F_2 offspring are lower than those between the pure-race parents and their F_1 offspring, despite certain elements in our procedure which would tend to increase correlation in the former

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[1] A similar calculation, based upon all of the characters of all of the *Peromyscus* hybrids thus far considered (76 pairs of variables) yielded a coefficient of $+0.204 \pm .074$. One of the series, the "Carlotta-Calistoga" lot, yielded, however, a negative coefficient (-0.17).

case. A conspicuous exception to this general rule is, however, to be noted in the case of foot pigmentation.

(5) In general, coefficients of fraternal correlation seem to be lower for the F_2 generation than for the F_1.

(6) Pairs of characters which are correlated in the parent races and in hybrids between these show, in general, a higher degree of correlation in the F_2 generation than the F_1.

(7) Certain elements which contribute to the general pelage color of these mice (aside from those which are obviously interdependent) are found to be correlated with one another, and thus either to have a common genetic basis or at least to depend upon factors which are more or less closely linked. On the other hand, another pair of characters which differ in the same direction in the two races (foot pigmentation and percentage of "black" in the pelage) are apparently not at all correlated within the single race, although individual differences in these characters are known to be partly genetic. Since these two characters are known to have varied together geographically in more than one species of *Peromyscus,* their coincident modification has probably resulted, in some way, from external agencies and not from correlated variations, due to internal causes.

Items 1 to 6, in the foregoing summary, bear upon the theory of "multiple factors," *i.e.,* the view that each of the subspecific differences here discussed is due to the cumulative effect of a number of independent genetic factors, whose effects, taken singly, are similar in their nature. Without entering into an extended discussion, it may be said that the facts here presented conform, on the whole, to the requirements of that theory, and that no facts narrated in the present paper constitute serious objections to it. Items 3, 4 and 6 represent findings which are somewhat at variance with those presented in certain recent papers by the senior author, based upon other hybrid series, and in large degree upon other characters than those here considered.

After careful consideration of the present series, and a further statistical examination of previous material, the senior author is prepared to admit that the case for a "multiple factor" explanation, while far from conclusive, is considerably stronger than he was earlier disposed to concede. It is important, however, that he should make clear just how much is involved in this admission.

The evidence renders probable the segregation, in the second hybrid generation, of unlike hereditary materials derived from the two parent races. It likewise renders probable the independent assortment of materials affecting the development of various single character differences. Furthermore, it seems to show that each one of these character differences, so far as it is hereditary at all, is influenced by a considerable number of units which segregate independently of one another.

On the other hand, there is nothing in the present evidence to support the view that the entire heritage of an organism is constituted by these segregating "factors." Nor would even a decisive demonstration of the segregation of hereditary units constitute a proof that the segregation was complete or that the units were unchangeable or incapable of influencing one another. One's beliefs in regard to these matters must depend upon the totality of evidence from all sources, and few would claim that at present this evidence is decisive in favor of any particular hypothesis.

In the present state of our knowledge, it is still permissible to suspect that the mendelian "genes" are not the only substances responsible for the hereditary differences between one organism and another. It has frequently been suggested that the genes play the rôle of enzymes in the process of development. But an enzyme must have a "substrate" to act upon, and the end result depends upon the properties of both. Such viewpoint is in harmony with the contention of Loeb "that the Mendelian factors of heredity must have the embryo to work on and that the organism is not to be considered a mere mosaic of Mendelian factors" ("The Organism as a Whole," p. 247).

The results of the present studies are not offered as evidence for such a modified or restricted mendelian viewpoint, though they are in no way inconsistent with it.

ADAPTIVE CHANGES IN SHADES AND COLOR OF *FUNDULUS.*

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I. Introduction.

Considerable attention has been paid to the changes in shade and color which fishes frequently undergo, and the significance of the phenomenon. The subject is of some theoretical interest and has given rise to a rather extensive literature, though the conclusions arrived at by the various authors are far from unanimous.

There are two important problems involved in these investigations, namely (1) whether fishes simulate the color of the environment and (2) whether they are able to discriminate light of different wave-lengths. In other words, is the spectrum objectively speaking the same for them as for a normal human being or do they see the spectrum as a color blind person does—a gray band of varied intensity. If fishes simulate the background, this indicates discrimination of wave-lengths or color vision, for it has been established that the eye and not the skin as a whole acts as the receptor of the light stimulus. It is, of course, here not implied that they have the same color sensations as a human being has.

Several investigators have attacked the problem of color vision more directly by the method of forming food-associations with definite colors and testing fishes for their ability to distinguish these colors from others of equal brightness. Van Rynberk (1906) gives a critical survey of the earlier work on color changes in fishes and in animals generally, in which the method of food association, as well as that of instinctive response and simulation of background, have been used in testing color vision. The following brief account of the more important recent literature indicates the results obtained by investigators on color changes in various species.

Zolotnitzki (1901) using *Cheironomus* larvæ as food until fishes were accustomed to it, next presented bits of wool of the size and shape of the larvæ but of different colors. The responses to the red were more frequent than to the other colors. He concluded that they discriminate colors, but he disregarded the brightness factor.

Schöndorff (1903) maintained that the earlier workers were in error in asserting that fishes assume the color of the background, but he did so on scanty evidence.

Van Rynberk (1906) found that the flatfish, *Rhomboidichthys* simulates the environment in a striking manner.

Washburn and Bentley (1906) noted that the horned-dace, *Semotilus atromaculatus* discriminates red, green and blue.

Reighard (1908) concluded from his observations on the responses of the gray snapper, *Lutianus griseus*, that it is able to discriminate colors.

Objections have been raised to the conclusion of investigators up to this time, that there was no certainty as to brightness of light for the eyes of the fishes.

Bauer (1910 *a*) experimenting with *Charax puntazzo, Atherina hepsetus L.*, *Box salpa* and *Mugil* sp. concludes that "Wie bei normalen Menschen tritt bei Helladaption, zur Unterscheidung der Helligkeit, die Unterscheidung der Farbenwerte." He observed aversion to red or "Rotscheu" in *Charax* and *Atherina*, and Purkinje's phenomenon in *Mogil* and *Atherina*.

Hess (1910), a leading investigator in the field of color vision in animals, maintains, however, that fishes see the various parts of the spectrum exactly as a color-blind person does at any intensity of light, or as a normal person does at lowered intensity. Bauer (1910 *b*) says this is true only of dark-adapted fishes.

Von Frisch (1911, 1912, 1913) carried out many exact experiments with *Phoxinus* and other species and has come to the conclusion that fishes discriminate colors as well as simulate the background. Hess (1913) maintains, however, that the color of the background has no influence on the color of *Phoxinus*.

Freytag (1914) also found no evidence of color simulation in *Phoxinus*, but subjected the fishes to the stimulus for only twenty-four hours.

Summer (1911) found that certain flatfishes simulate the background not only in shade and color but also in pattern and his photographs given ample evidence that at least these species do so.

Mast (1916) in a very detailed study of the flounders *Paralichthys* and *Ancylopsetta*, proves that these species simulate the background in pattern as well as in shade and color and gives excellent autochromes illustrating the colors assumed.

Reeves (1919) using the method of food association and unlearned responses, and equating the light intensities, concludes that the sunfish (*Eupomotis gibbosus* L.) and horned-dace (*Semotilus atromaculatus*) discriminate light of longer wave-lengths from light of shorter wave-length and from white light, while Ohashi (1921) finds in support of Hess that goldfish and carp are unable so to discriminate, their responses being caused by different light intensities.

During the winter of 1922–23 experiments on *Fundulus heteroclitus* were carried out at the Zoölogical Laboratory, Harvard University, giving positive results which we shall now describe.

II. Adaptive Changes in Shade.

Parker and Lanchner (1922) have recently made tests to ascertain the effect of illumination on the shade of *Fundulus*. They found that the fishes when placed in a white environment represented by a box lined with white paper and illuminated by an incandescent lamp, were of a light shade; those placed in a black environment represented by a box lined with black paper, were of a dark shade; while those placed in absolute darkness were, contrary to what one would expect, of a light shade. It was also observed that when temporarily blinded they did not exhibit any change in the various environments, showing that the action of light was not a direct one, but indirect, the eye being the receptor.

As a starting point these experiments were repeated and uniform results agreeing with these were obtained.

A test was now made to determine the effect of different colored backgrounds on the shade of *Fundulus*. Four square boxes were lined each with a differently colored paper, one side being left open for the entrance of light. The colored papers matched those named in brackets as given in Ridgway's "Color Standards and Color Nomenclature." The first was lined with light yellow paper (Lemon yellow, Pl. IV., No. 23); the second with red (Nopal red, Pl. I., No. 3. i); the third with green (Scheele's green, Pl. 6., No. 33. i); and the fourth with blue (Bradley's blue, Pl. 4, No. 51).

Fishes were first made to assume the light shade by being left for several days in a white glazed earthenware vessel in the laboratory. From this stock they were selected for experiment. Four specimens were placed in each of four battery jars filled with water to a depth of 10 cm. and the jars placed in the colored boxes. The boxes were illuminated by a white Mazda lamp (100 watts) placed at a distance of 60 cm. from the jars. For purposes of direct comparison all combinations of colored boxes taken two at a time were made, the two boxes and incandescent lamps being screened off from each other. The experiments were all carried out in a large dark room, so that the fishes in each box were under the same environmental conditions, except difference in background. The tests were each of 24-hours duration.

It was found after repeated experiments that the specimens from each colored background showed a distinct shade. When compared on a white ground in diffuse daylight, the specimens in the yellow box showed the lightest shade, those in the red box came next, the specimens in the green box were darker and in blue darkest. No change of color was noticed. All four were subject to the same light energy from source, but obviously we cannot conclude from this, that quality of light and not brightness of background, determined the effect. These tests of relatively short duration, seemed rather to indicate that brightness and not color or wave-length determined the shade. Further tests were required to settle the point.

In the next series of experiments, the effect of different monochromatic lights on fishes placed in white and black lined boxes was tried. For this purpose Wratten filters were used. These were of the larger size, namely three inches square, the smaller size which were first used not supplying sufficient light surface at the selected distance to illuminate fully the boxes. The filters selected were: Yellow (K, No. 6) Red (F, No. 29) Green (B. No. 68) and Blue (No. 45), approximately corresponding to the four colored backgrounds used in the preceding tests. The Mazda lamps were placed in boxes covered with black paper except the space occupied by the filter set in the middle of the front side. In front of the filter about 60 cm. distant, two boxes were set up, one being lined with dull white and the other with dull black paper, each being so placed as to receive equal amounts of light. All shadows were avoided.

Screened off from this apparatus, a similar one was set up in the same large dark room, so that the effect of each of two monochromatic lights on two different backgrounds could be compared under identical conditions. Each test was of 24-hours duration and comparisons were made on a white ground in diffuse daylight.

When the specimens placed in the white lined boxes were compared, they showed the same sequence in shade as in the previous set of experiments, namely from light to dark; yellow, red, green, and blue, though due to less intensity of light by use of filters, the effect was less pronounced than with the white light on variously colored backgrounds. When the effect of the same monochromatic lights on specimens in the white-lined box was compared to the effect on those in the black-lined box, it was observed that in each case the latter were darker in shade than the former. And moreover little or no difference could be observed in the degree of darkness shown by the specimens in the black-lined boxes illuminated by different monochromatic lights. The different monochromatic lights have the same effect as white light on a black background.

Parker and Lanchner (1922) suggest the hypothesis that contrast rather than simple vision is involved in responses of this kind. Keeble and Gamble (1904) state that the shade assumed by crustaceans depends on the ratio of light received by the eyes directly from the source, and that received after reflection from the background. Summer (1910) believes that this ratio holds good for fishes. Mast (1916) carried out experiments to test this hypothesis and concluded that while simulation of background is not controlled solely by light reflected from the bottom, the results of the experiments "throw considerable doubt on the hypothesis."

We have made no tests to determine whether the shade depends on this ratio. The experiments merely indicate that the background largly determines the shade. The slight differences in shade of those colored by different monochromatic light is probably due to different amounts of light passing through the filters, and that lights of all colors had a stimulating effect just as white light in causing partial or total contraction of the black pigment cells or melanophores.

From these experiments the conclusion cannot be drawn that it is quality and not quantity of light that determines the shade. The melanophores expand and contract with comparative rapidity in light of different intensities, and as no adaptive coloration was observed in these 24-hour tests the shade assumed was more probably regulated by the different intensities of the various monochromatic lights.

III. Adaptive Changes in Color.

A. Effect of Colored Backgrounds.

In order to test whether *Fundulus* simulates the background in color as some other species had been shown to do, the fishes were placed in battery jars and set in boxes lined with the same colored papers as in the first series of experiments. Here, however, the boxes with the contained fishes were placed with the open side facing the windows of the laboratory and subjected to the various color stimuli for several weeks. The water was renewed daily, and the temperature never varied more than a few degrees, remaining between about 11 and 15° C. so that the specimens were at all times under the same environmental conditions except that of color. In each jar there were 5 fishes each about 8 cm. in length. On the second days it was noticed that the shade was distinct for the fishes in each of the colored boxes, the sequence from light to darker in shade being yellow, red, green and blue. When these experiments were repeated, it was observed that these differences in shade could be recognized within three or four hours. On the sixth day there was also noticed a distinct color for each group of fishes in the four colored backgrounds. The group in the yellow box were decidedly yellow; those in the red showed a pink color; those in the green a more pronounced green than that sometimes shown by normal specimen, the region above the eyes revealing this tint in a striking manner; while those in the blue box were of a gray, slate-blue color. The adaptation in color increased during the second week when they reached their maximum degree of simulation and maintained it as long as the experiments continued, namely, for about six weeks. This was approximately the maximum time that the specimens could be kept in normal health under the artificial conditions in the laboratory and following this they gradually died. The tests were repeated many times between November 1922 and June 1923 with different sizes of fishes ranging in length from 5 to 10 centimeters and uniform results were obtained. Fundulus simulates the backgrounds after a prolonged stimulation and the effect produced cannot be due to intensity of light alone.

For purposes of comparison, fishes were also placed in three other boxes without color; one lined with dull white paper, a second with neutral gray and a third with dull black paper. Those in the white box showed the pale tint of light-adapted fish; those in the black box a very dark shade, while those in the gray showed an intermediate shade. On the other hand the fishes in the colored boxes each showed a color distinct from the others and from those in the uncolored boxes. The effect, then, cannot be due to intensity of light reflected on the background, but must be due to the color stimuli. Otherwise there is no explanation of the fact that fishes in the yellow box showed a decidedly yellow color while those in the white box which reflected all light, were pale. Although the melanophores or black pigment cells by their contraction or expansion due to intensity, indirectly influence the color to some extent by exposing or covering other color elements of the skin, the resulting effect of each colored background cannot be explained by this factor. The specific effect is due to color or wave-length.

It was first proved by Pouchet (1876) and later by von Frisch (1911) and other investigators that the expansion of the chromatophores is controlled by the sympathetic nervous system; the eyes act as receptors for the stimuli that cause changes in shade and color. For when the fish were blinded, no adaptive change in shade or color took place. In the experiments on Fundulus, collodion made opaque with lampblack was placed over the eyes and held by stitches of silk thread sewed into the superficial skin. These collodion caps generally remained on for a few days, sufficiently long to show that no adaptive change in shade takes place when fish are blinded, but not long enough to show that adaptive coloration is likewise controlled through the medium of the eye, as has been established by investigators for other species. Other methods were employed but the shock of operation caused changes in color and shade which made the specimens useless for purposes of safe comparison.

Finally it was found that making the cornea opaque with a heated needle had no perceptible influence on the healthy condition of the fishes. Specimens so treated were allowed to remain for a couple of days in order to make sure of their otherwise normal condition before being placed with other fishes in the differently colored backgrounds. In each case the blinded animal showed no simulation of the background.

It is an error to assert, as has been done by some, that blindness necessarily causes maximum darkening of the fish. It depends on the method and length of operation, and different degrees of darkening can be obtained almost at will. The possible objection that making the cornea opaque might still permit the eye to receive the stimulus and thus account for lack of darkening is met by the fact that fishes thus blinded maintain a light shade if placed in that condition on a black background, which they would not do if the eye were still functioning.

Spaeth (1913) has shown that light, temperature, and also various salts have a direct effect on the contraction of the chromatophores, both melanophores and xanthophores, when the scales with the superficial chromatophores are separated from the fish. But in our experiments with the normal fish, these factors did not influence the results, as the conditions, except background were uniform for all. Sečerov (1909) maintains that the light has a direct action, but this seems disproved by von Frisch.

B. Effect of Spectral Lights or Different Wave-lengths.

The eye being the receptor of the light stimulus, the results of the above experiments can only be explained on the assumption that *Fundulus* objectively discriminates light of different wave-lengths. Since, however, intensity apparently plays an important rôle in the different shades assumed by the fish and to some extent in color, by the relative degree of expansion of the melanophores thus covering or exposing other color elements, a more rigid test was made by using spectral lights of different wave-lengths but of the same intensity.

In much of the work done on color vision in lower animals where the method of food association with definite colors was used, the factor of intensity was either totally disregarded or arrived at only approximately by comparing the relative brightness of the differently colored objects presented. This method of comparison being subjective, can give no exact quantitative data. Filters whether liquid or glass, are likewise unsafe as they are not always monochromatic, nor do they always exclude the infra-red and ultra-violet rays, and of course differ considerably in the amount of light which they allow to pass through. It is therefore necessary that either the brightness of the various spectral lights should be equated by the use of a flicker photometer, as Reeves (1919) has done in food association experiments, or the lights, varying in quality, should be made equal in the quantity of radiant energy. This latter method was first employed by Laurens (1911) at the suggestion of Prof. G. H. Parker.

More recently Laurens and Hooker (1917) have described an apparatus by means of which twenty-three lights were obtained each thirty wave-lengths in width but equal in radiant energy and extending by steps of ten from 420$\mu\mu$ to 670$\mu\mu$. The Hilger deviation spectrometer was used to obtain spectral lights and the radiant energy equated by means of a thermopile and galvanometer, thus determining the distance of the lamp from the slit of the collimator, in order to give, equal energy for all sets of lights.

This apparatus was used to test the effect of spectral lights on the color of *Fundulus*. It was necessary, however, to select only a few parts of the spectrum. In fact only two sections would be strictly necessary, one of long wave-length the other of short wave-length. Since the spectral lights are of equal energy, the fishes should show the same effect if intensity is the sole factor. If the effect varies in different spectral lights, it can be due only to wave-lengths. Using the data provided by Laurens and Hooker, the following spectral lights with corresponding conditions in the apparatus were selected.

Range of Wave-lengths, in $\mu\mu$.	Position of Drum.	Width of Slit, Mm.	Range of Wave-lengths Either Side of *D* Line.	Distance from Lamp to Slit in Cm.
470–500	484.0	3.4	561–622	4.0
520–550	534.0	2.3	569.5–622	13.0
560–590	574.5	1.8	573.5–606	19.0
620–650	625.0	1.3	577–601.5	28.5

For a light-source, a 1000-watt Mazda lamp was used. The lamp was placed in an iron box 12 inches square at the base and 20 inches high. On the front of the box an aperture was made for light. To secure ventilation apertures were made below in front and near the top of the rear side; these were screened by sheets of asbestos. An asbestos funnel was also placed between the aperture of the lamp box and the slit of the collimator. A metal cylinder lined with black paper and closely fitting to the ocular of the telescope conveyed the light to the jar containing the fishes, placed at a distance of 60 cm. The jar, measuring 10x5.5 cm. and 15 cm. high was covered with white paper on all sides except that exposed to the spectral light and screened off from any light leakage from lamp box. The apparatus was set up in a large dark room, so that no light other than that passing through slit of the spectroscope could enter the jar. As the light band of 30 wave-lengths is quite narrow in the red end of the spectrum, the narrow side of the jar faced the light. Four fishes about 5 cm. long were used in each test. The water was renewed by siphoning and kept at room temperature. Each test ran day and night from Monday to Saturday, when fish were examined.

In the first test, in which the fishes were exposed to the blue light waves between 470 and 500$\mu\mu$ in length, the pronounced blue color observed in specimens subjected to prolonged stimulus of blue background as noted above, was absent, the specimens not differing in any noticeable degree from some normal fish. The second test (green) was likewise inconclusive. This is the most difficult change to verify, for in light adapted fish, a green tint is always present. The third test, that of yellow—560 to 590$\mu\mu$—gave a decided response, all specimens becoming yellow. It likewise gave a basis for judging the preceding tests. For if intensity alone is a factor in the color changes, since it was equal in all the tests, the specimens in the first two should show the same yellow color as in the third. The experiment was repeated with the same fish after they had again assumed the pale tint by being placed on a white background and the response to yellow occurred again in about 36 hours. Repetition, as observed by Mast and others, increases the rapidity of the response. The fourth tests, that of red which included wave-lengths 610 to 640$\mu\mu$ also called forth a yellow response but less pronounced than in the case of yellow rays.

It is worthy of note that in all tests with spectral light of equal intensity, the shade was about the same, the melanophores being contracted. From this it may be concluded that light of any color has a stimulating effect like that of white light in causing contraction of the melanophores. It shows clearly that intensity of light plays an important rôle in the expansion or contraction of the melanophores and thus indirectly affects the colors of fishes by exposing or obscuring other color elements. Nevertheless, the fact that the yellow part of the spectrum caused a decisive change to a yellow tint by expanding the xanthophores or yellow pigment cells, while the intensity remained the same as in other spectral lights to which the fishes were exposed, is definite proof that in *Fundulus,* the quality or wave-length has a specific effect apart from quantity of light or intensity. That the responses to the various colors is here not so pronounced as in experiments where fishes were placed in colored boxes and exposed to diffuse daylight for several weeks, is due to the fact that the fishes were exposed to spectral lights for only five day periods. Moreover, the area and brightness of the colored environment were much reduced.

IV. THE BASIS OF ADAPTIVE CHANGES IN SHADE AND COLOR.

A. The Normal Coloration.

The specimens freshly taken from the sea varied greatly from a yellowish green to black when seen from above. The silvery bars of the adult male with scattered yellow and bronze colored spots are very conspicuous on specimens otherwise dark. The bars are due to the smaller number of melanophores superficial to the scales and to the presence of dense groups of guanophores or iridocytes, which are frequently arranged like branched filaments. The guanophores are highly refractive and when viewed by reflected light, reveal the whole gamut of colors.

As previously stated all specimens were made to assume the pale yellow green tint by being placed for several days in a white dish before being subjected to the various other colored environments. If, now, the living specimens be examined by reflected light under a microscope or with a good hand-lens, it will be seen that there is more than enough material to work on to account for the colorations revealed in the various experiments. The most striking phenomenon is the presence of brilliant points scattered over the dorsal and lateral surfaces. This is especially prominent when direct sunlight is allowed to fall on the fish. The bright points occupy the central portion of many melanophores and in their dark setting appear like jewels. The phenomonon in due to an association of iridescent guanophores or iridocytes with melanophores.

For further detailed study of the color elements, fresh specimens from each of the colored environments were fixed by dipping them in hot water for about 10 seconds, then in cold and finally examining them in glycerine. When examined under higher magnification, the guanophores are seen to be spindle-shaped bodies having an average length of about 20μ and a maximum breadth of 3 to 4μ. There are other smaller guanophores or iridocytes, ovoid in shape being about 11μ in length and 3 to 4μ in width. These iridocytes are especially found in patches apart from any association with melanophores and give rise to the mottled condition of the male. When associated with melanophores, the guanophores seem to acquire a more brilliant iridescence than when they occur separately. The more common colors reflected in the specimens adapted to a light background are yellow, orange, and green. If the scales are removed, and examined under the microscope, these iridescent points are not to be seen in the melanophores, which remain on the distal portion of the scales after removal. They are located on the proximal portion of the scale and the reflected rays pass through the distal portion of the overlapping scale.

It is well known that many animals show a green coloration though there is no green pigment present. This is true of *Fundulus*. The green tint is due to the combination of the effects of melanophores and xanthophores over a layer which reflects blue or over iridocytes possessing that physical property which Pouchet termed "le cerulescence." When paraffin sections are made through the skin there is to be seen a compact layer of cells occupying the inner part of the dermis next to the muscles. The cells are rectangular in shape with their long axis parallel to the surface. The nuclei are likewise elongate. Cunningham and McMunn (1893) have suggested an ontogenetic relation existing between this layer which they term the argenteum, and the iridocytes.

There is also a close relation in structure and optical properties between the guanophores of the integument and those of the peritoneum. The peritoneum of *Fundulus* is covered with dense black pigment on the surface exposed when the body cavity is opened, and the inner surface shows the typical silvery sheen. This layer is more highly specialized than the reflecting layer of the integument. It does not stain with anilin dyes. If the pigmented surface be examined by deflected light, the same phenomenon of brilliant points associated with melanophores is seen.

showing that the guanophores have the same optical property as was present in the integument.

B. Shade Adapted to White, Gray, and Black Backgrounds.

As is well known for many species, changes in shade are due to contraction or expansion of the melanophores. Ballowitz (1893) has shown that the chromatophores of fishes are innervated by branches which proceed from a dense nerve-net surrounding the chromatophores, or even direct from a nerve bundle itself. These constitute the true pigmento-motor nerves. According to Ballowitz, they have many free terminations on and within the chromatophores. As already pointed out, the eye acts as the receptor and the stimulus is conveyed by way of the sympathetic nervous system to motor fibers innervating the chromatophores as Pouchet surmized and von Frisch clearly demonstrated. Von Frisch (1912) maintains that a centre for contraction of the melanophores exists at the anterior end of the medulla.

On a white background the melanophores and xanthophores are contracted. On a black background the melanophores are expanded and the xanthophores are contracted. In the male specimens the silvery bars and the bright mottled condition of the lateral portions are retained no matter how long the fishes are left in this environment. In fact, they are more pronounced by contrast with the dark ground-color of the fish. No evidence of a reduction or increase in the number of melanophores after a prolonged stimulus in the white or black environment was observed, such as Kuntz (1917) asserts to be the case in *Paralichthys*. On a gray background the specimens were intermediate in shade, the melanophores being partially contracted.

C. Yellow Adapted.

The melanophores in specimens exposed to a yellow background for several weeks were maximally contracted. The xanthophores on the other hand were maximally expanded, to such an extent that they appeared diffused and the limits of the individually pigment cells were difficult to determine. In this condition, the blue reflecting layer is largely concealed. Examined by a reflected light, the brilliant points on the scattered melanophores are very striking, the predominant colors reflected being yellow and orange.

D. Red Adapted.—Vaso-dilation.

The melanophores on the superficial distal portions of the scales were likewise contracted in fishes exposed to a red environment for the same period as in the preceding test, but those in the proximal portions and in deeper parts were not fully contracted. The xanthophores, however, were not fully expanded as in the yellow adapted fish. There was a denser spherical concentration of the pigment giving an orange tint. There are no red pigment cells in *Fundulus*. But the color of the specimens adapted to a red background, was quite distinct from that in the yellow. Instead of the bright yellow of the latter they showed a pink color. It was at first thought that this might be caused by the partially contracted xanthophores giving a deeper yellow or orange to the cells, in combination with the melanophores. On further examination it was seen that the dorsal region of the head, usually pale, was quite red due to the dilated condition of the blood capillaries. The opercular region showed a striking network of vessels. The capillaries of the trunk were likewise dilated giving a pink color to the fish. Before the melanophores are more fully contracted by the prolonged stimulus, the color is brownish.

The pink color is without doubt, due to the blood in the capillaries. As in the cases of experiment with fishes on other colored backgrounds which ran concurrently through the winter months, the results here were always the same. The only exceptions were those fishes taken late in the spring which did not seem normal and were affected by the sudden change in temperature on being taken from the sea. Many of these died and those that lived were exceedingly slow in assuming the light shade when placed in a white vessel before being exposed to the red environment. And strangely enough, though not revealing any blood vessels over trunk and head, the caudal find of one of these specimens was very red, due to vaso-dilation. The general coloration of these late-spring specimens on a red background approached the yellow, but was more sombre. The fact that in these specimens lacking the pink coloration, the blood capillaries were not visible, as in other specimens on a red background, confirms this explanation.

While the distinctive color is then due to the blood in the capillaries, there still remained the possibility that the cutaneous vessels were equally dilated in fishes adapted to other environments, but that the vessels were covered with melanophores. This seemed to be true of the blue adapted fish, for the dorsal region showed dark lines as if the melanophores were especially abundant and expanded over the capillaries. But the anterior portion of the head, where few melanophores are present, failed to show any dilation. Again, the fishes adapted to a yellow background where melanophores are maximally contracted and likewise those adapted to a white background where both melanophores and xanthophores are contracted, showed no evidence of vaso-dilation.

E. Green Adapted.

In those adapted to the green environment, the shade was darker than in the preceding cases, due to partial expansion of the melanophores. The xanthophores were only slightly expanded. The most striking portion of the body in which this color was emphasized, was above the eyes.

F. Blue Adapted.

In these specimens the melanophores lying in the deeper portions of the integument, and those lying upon the proximal portion of the scales were expanded, thus giving a grayish tone when seen through the overlying adjacent scales. On the distal portion of the scales, the melanophores were generally contracted. The xanthophores were maximally contracted thus exposing the blue reflecting layer. When the surface of the skin was examined by reflected light, the predominant color coming from the brilliant points was a bluish green.

V. Discussion

From the brief outline at the beginning of this paper of the more recent work on the problem of simulation of fishes to the background, it is evident that the majority of investigators have found that the various species tested, do simulate the background and discriminate light of different wave-lengths. To these species, *Fundulus* can unquestionably be added.

Mast (1916) who made a detailed study of two species of flounders and observations on other fishes kept in the aquaria at

Beauford, comes to the general conclusion that "adaptive changes in shade occur in the skin of practically all of the different fishes in the region of Beaufort, N. C.; adaptive changes in color in many, but adaptive changes in pattern in only few."

When the problem of color discrimination is attacked directly by the method of unlearned responses or by forming food associations, the factor of light intensity must be the same for all wave-lengths. Hess, who has done most in the field of color responses in animals generally, stoutly denies that conclusive proof has been brought forth for color vision in fishes, and Parsons (1915) accepts Hess's conclusion.

One benefit of this criticism has been to bring about the use of more precise method in testing the responses of fishes to different colors. Reeves (1919), however, equated brightness for different colors and concluded that fishes discriminate light of different wave-lengths. On the contrary, Ohashi (1921) concludes that intensity alone is responsible for the different responses in the goldfish and carp when subjected to monochromatic light. As this is the most recent paper that has come to our notice, and as the author arrives at conclusions different from those stated in the present paper, we shall discuss Ohashi's work in detail.

Ohashi first noticed that goldfish up to three years old were attracted to different colored lights when subjected in turn to these stimuli. In other words they are positively phototropic to all monochromatic lights.

In the following experiments red and green liquid filters were set in the top of an aquarium equidistant from the ends and the lamp placed midway between them. The fish assembled under the green light which was the brightest for them. When the lamp was adjusted so that the green and red appeared of equal brightness to the observer, the fishes assembled in about equal numbers under each color. From these observations Ohashi concludes that no support for the belief that fishes have color vision can be found here. Apart from the fact that the liquid filters admittedly did not give pure monochromatic lights, there is no certainty that the apparent equality of brightness of the two lights to the human eye were likewise of equal brightness to the eyes of the fish. In short, brightness cannot be objectively determined by simply comparing the different colored lights and no valid conclusions can be drawn from tests of this kind.

In his food association experiments, Ohashi noted that fishes could not discriminate red when this color was plainly visible to the human eye. This does not disprove color discrimination in fishes for it is quite probable that at a lowered intensity they would be unable to discriminate color and yet be able to do so above a certain threshold of intensity. Hess, in fact, has shown that the red end of the spectrum is shortened for fishes.

When subjected to prolonged stimulation by red and by violet lights the fish became reddish yellow and bluish and on examination it was found that the melanophores expanded in blue light and the xanthophores in yellow light. Unless, therefore, there is a direct effect of light on the chromatophores as Šecerov claimed, but which von Frisch and Mast deny, the reported adaptive coloration must be due to color discrimination.

Ohashi next placed fishes on eight bottoms of colored paper; blue (two tints), red (also two tints) white, black, and gray (two shades). Changes in color were noted in 30 seconds and completed in two minutes, the principle colors noted being dark, reddish yellow and blue. If we have here a case of adaptive coloration, it would indicate a remarkable rapidity in the response. Obviously it is only adaptation in shade, as the author finally concluded when all specimens were compared with one another and it was noted that those which had been on a dark red bottom were similar in shade to those on a grey. The period was entirely too short, however, to conclude from this experiment that they are unable to simulate the background in color.

The same may be said regarding his final experiments where fishes were subjected to the stimulus of red and blue from filters, those in the red becoming reddish yellow and those in the blue, dark within five minutes. When the blue light was made more intense than the red, the fishes in the blue light became yellow and those in the red dark. Again intensity of light was no doubt the factor causing different degrees of contraction of the melanophores as the author concludes and as we have noted for *Fundulus*. This however, does not disprove color discrimination for these species. On the contrary, the results following prolonged stimulus of different colored backgrounds indicate that the species tested do simulate the background.

The subjective significance of the phenomonon of color simulation. is not considered here. It is important first to know, as Uhlenhuth (1911) pointed out, whether lights of different wave-length bring about different responses. This has been found to be true of Fundulus heteroclitus.

This research was suggested to me by Prof. G. H. Parker and carried out under his supervision. To Prof. Parker I am indebted for many helpful suggestions, for the privileges of the Harvard Laboratory and for many other courtesies extended while making this investigation.

VI. Summary

1. Adaptive changes in shade occur in *Fundulus heteroclitus* when placed on white, gray, and black backgrounds. This adaptation is brought about by the contraction or expansion of the melanophores.

2. On colored backgrounds for short periods, adaptation in only shade occurs.

3. Prolonged stimulation by colored backgrounds brings about adaptation in color. This occurred in yellow, red, green, and blue environments.

4. The experiments with spectral lights of different wave-lengths but of the same radiant energy or intensity, show that the melanophores respond similarly at all wave-lengths, the contraction being due to light intensity.

5. The response to the longer wave-lengths, red and especially yellow, in causing an expansion of the xanthophores, though intensity was the same as in the blue or short wave-length end of the spectrum, shows that the quality of light or wave-length has a specific influence on the coloration of *Fundulus*.

6. The stimuli causing changes in shade and color are received through the eyes.

7. The changes in color are brought about by the degree of expansion or contraction of the melanophores and xanthophores combined with the optical properties of the guanophores or irido-

cytes and the reflecting layer. The guanophores are frequently found associated with the melanophores.

8. Adaptive change to a yellow background is brought about by a maximum expansion of xanthophores and a maximum contraction of the melanophores.

9. In fishes adapted to a red background, the xanthophores are only partially expanded and the melanophores contracted. The pink color is due chiefly to a dilation of the blood capillaries.

10. In fishes adapted to a green background, the green tints present in the normal light adapted fish, is increased and is especially noticeable above the eyes. The xanthophores and melanophores are partially expanded.

11. In blue adapted fishes the xanthophores are maximally contracted and the melanophores in the deeper portions of the dermis and upon the proximal parts of the scales, are expanded.

Postscript. Since the preparation of this paper I have had the opportunity of repeating the experiments on *Fundulus* at the St. Andrew's Biological Station. My results have been the same as those obtained in the Harvard Laboratory except that I failed to get on a red background the pink coloration and vaso-dilation described in this paper. Whether this is a seasonal or a local difference in the fishes remains to be worked out.

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BIOLOGICAL BULLETIN

PHYSIOLOGY OF THE ORTHOPTERA.

Hydrogen Ion Concentration of the Blood and Alimentary Tract of Certain Orthoptera (Grasshoppers).

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Recent investigations on the hydrogen ion concentration of blood and body fluids have dealt almost exclusively with higher vertebrates and particularly with mammals. Little information seems to exist for lower forms, especially the insects. Crozier (1) in recent work with transparent aquatic insect larvæ has strikingly shown the hydrogen ion concentration of different parts of the alimentary canal, while Bishop (2) has pointed out the hydrogen ion concentration of blood in the larva and pupa of the honey bee. Jameson and Atkens (3) have studied the hydrogen ion concentration of the blood and alimentary canal of the silkworm. Since no such information seems to exist for grasshoppers it was thought desirable to study the blood and alimentary canal of these forms. The results herein presented are based upon large numbers of animals comprising seventeen different species.

The method employed in determining the hydrogen ion concentration (pH) was a colorimetric one, essentially similar to that described by Felton (4). Various dyes of the Clark and Lub (5) series were used and in all cases different dyes were employed in checking results so that possible errors due to the color of the blood or body fluids could be eliminated. Blood was collected from the body cavity under oil in capillary pipettes as well as directly from an incision in the body wall. No marked differences in pH values of blood collected with and without exposure to air were noted when determinations were carried out within one minute. The alimentary canal was usually taken out

Species.	Blood.	Fluid from mouth.	Crop.	Cæca.	Stomach.	Int. and Rectum.
Melanoplus differentialis	6.81 (6.4–7.0)	5.60 (5.2–5.8)	5.81 (5.5–5.9)	6.06 (5.8–6.9)	6.52 (5.6–7.5)	6.43 (5.4–7.4)
Melanoplus f. rubrum	6.79 (6.7–6.86)	5.1 (4.9–5.3)	5.37 (5.1–5.6)	6.23 (5.8–6.9)	6.55 (5.9–7.3)	6.20 (5.6–7.3)
Melanoplus bivittatus	6.35 (6.0–6.7)	5.45 (5.4–5.6)	5.60 (5.3–5.8)	5.98 (5.8–6.2)	6.40 (5.8–7.2)	6.58 (5.7–7.5)
Melanoplus scudderi	6.80 (6.6–6.9)	....	5.43 (5.0–5.9)	6.23 (5.9–6.9)	6.66 (6.1–7.2)	5.86 (5.8–5.9)
Dichromorpha viridis	6.70 (6.4–6.9)	....	5.62 (5.5–5.8)	5.96 (5.8–6.2)	6.16 (5.8–6.4)	6.28 (5.8–6.6)
Arphia xanthoptera	6.83 (6.7–7.0)	....	5.6	6.03 (5.9–6.02)	6.46 (6.2–6.6)	6.40 (6.0–6.8)
Encoptolophus sordidus	6.77 (6.7–6.9)	5.75 (5.7–5.8)	5.72 (5.6–5.8)	6.25 (5.9–6.4)	6.58 (5.9–7.3)	6.32 (5.8–7.3)
Dissosteira carolina	6.85 (6.8–6.9)	5.40	5.75 (5.6–5.8)	6.05 (6.0–6.1)	6.30 (6.2–6.4)	5.95 (5.8–6.1)
Trimerotropis maritima	6.80	5.80	5.50	6.00	5.90	6.00
Scudderia furcata	6.70	....	6.00	5.90	6.10	7.30
Conocephalus ensiger	6.70	....	6.00	6.00	6.10	7.40
Microcentrum retinervis (?)	6.30	....	6.00	6.10	6.30	6.40
Shistocerca americana	6.60	....	5.40	5.75	5.80	5.75
Orphula pelidina	6.75	....	5.65	6.75	6.50	5.75
Spharagemon bolli	6.80	....	5.40	6.00	6.00	5.80
Chortophaga viridifasciata	6.80	5.30	6.00	6.90	6.95	6.80
Chortophaga australior	6.80	5.35	6.10	6.90	7.00	6.50

of the animals entire; washed and then split lengthwise so that food particles could be removed. Sections as well as the entire alimentary canal were then tested with the various indicator solutions. When the entire alimentary canal is left in the indicator solution for a short time and then examined under a binocular microscope with strong illumination the differences in pH values of the various parts are strikingly brought out. Animals starved for various periods of time, during which the alimentary canal was completely emptied of food, gave pH values similar to those found for normal individuals.

The accompanying table shows the average as well as the range in pH values for the blood and alimentary canal of the species examined. The average pH value for blood of grasshoppers seems to be 6.8. The dark brown fluid usually emitted by the animal when handled is quite acid in reaction. This fluid is doubtless made up of regurgitated substances from the crop as well as salivary secretions. The crop is generally quite acid in reaction while the blind pouches or cæca seem always to be less acid than the crop. The stomach or that part of the alimentary canal into which the cæca open, varies to a considerable extent in its reaction, being at times quite acid and at other times neutral or alkaline. The middle and posterior end of the stomach are usually less acid than the anterior end. The intestine and rectum, that part of the alimentary canal posterior to the point of entrance of the Malpighian tubules, also varies considerably in its reaction. In some species a rather marked alkaline condition is noted while in others both acid and alkaline reactions are found.

The pH of the blood of grasshoppers is of interest inasmuch as it is approximately the same as that found by other authors for different insects, *e.g.*, honey bee (Bishop 2), silkworm (Jameson and Atkens 3). It would be of considerable interest to know if insect blood functions in the transportation of gases, etc., as in the case of mammalian blood. Some phases of this question have been recently discussed by Bishop (2). Muttkowski (6) in studies on the respiration of insects has pointed out that the blood reacts with the oxidation tests for hæmoglobin regardless of the species of insect. These tests (guaiac, P-diamino-benzene, a-naphthol) have been repeated on grasshoppers used in the present experiments and positive reactions obtained only in the alimentary canal. As a matter of fact no tissue outside of the alimentary canal was found to give a positive reaction. The reaction in the alimentary canal seems to be confined to the cæca and stomach. It thus seems that for grasshoppers' blood other data are quite desirable before definite respiratory functions, similar to those for mammals, can be definitely attributed to it.

SUMMARY.

The blood of seventeen different species of grasshoppers tested by means of appropriate indicators shows an average pH value of 6.8. The average pH values for different parts of the alimentary canal are: fluid emitted from mouth 5.5; crop 5.7; cæca 6.2; stomach 6.4–7.4; intestine and rectum 6.4–7.2. Oxidation tests for hæmoglobin (guiac, P-diamino-benzene, a-naphthol) have been obtained with the alimentary canal and with no tissues outside of the alimentary canal.

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A SUGGESTION AS TO THE EFFECT OF THYROID GLAND SUBSTANCES ON PROTOPLASM IN GENERAL.[1]

ROBERT A. BUDINGTON.

Of the numerous angles from which the physiology of the thyroid gland has been approached, that of its influence during embryological development has been frequently studied. It is quite unnecessary for the purposes of the present paper to review the large literature of the subject; bibliographies are abundant. Briefly, one may recall that the effect of administering thyroid material to children is well known; Gudernatsch ('12), followed by many others, showed the precocious differentiation which follows the feeding of thyroid gland to tadpoles of Amphibia; Kunkel ('18) noted the response of flesh-fly larvæ to thyroid feeding; its influence on other phyla of animals has been investigated, even the Protozoa having been shown to react with an accelerated rate of metabolism, many structural modifications, a faster rate of reproduction, etc., to a thyroid diet.

In all instances of experimental research the reacting substance is, of course, protoplasm. Its condition, highly organized into the various specialized tissues, studied, experimented upon, and interpreted *en masse*—these facts, and our habits of thought, too often lead us into temporary forgetfulness of *protoplasm* as the fundamental and only vital substance in the material being studied.

As intimated above, it would seem that so many animals from such distinct levels of organization have shown clear responses to absorbed thyroid materials that we may easily conclude that something in thyroid composition affects and modifies animal protoplasm perhaps whenever and wherever it occurs. Correlated with this semihypothetical conclusion, one reflects that there is another large world of protoplasm identified with the plant kingdom; and the question arises, "Is *all* protoplasm enough alike so that that of plant associations, although of course never normally exposed to thyroid influences, is, never-the-less, susceptible to them?" If so, then the interest which attaches to the physiological effect of this endocrine substance is much increased; if not, then one of the differences in their characteristics, which perhaps was instituted when animal and plant protoplasms began their long evolutionary divergence, is made roughly apparent.

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[1] From the Department of Zoölogy, Oberlin College. The writer wishes to express appreciation of laboratory accommodations and numerous courtesies shown by the Department of Zoölogy at Columbia University during the latter part of this investigation.

A further question which arises is: "Are animal and plant protoplasms sufficiently alike so that, if both are susceptible to thyroid materials, they both react in the same manner?" That they apparently do to some infections has been fascinatingly brought out by the investigations of Smith ('20) in his scholarly studies into the nature of crown gall in plants, and its close similarity, etiologically, to human cancer. Very numerous instances of similar or comparable responses of plants and animals to the same physical stimuli could be cited.

The fact which has been taken as a point of departure in the present study is the use of thyroid tissues or extracts in hastening differentiation of vertebrate larval cell-masses into adult-like organs, and the provoking of a general precocious metamorphosis. The query, then, is: "Can thyroid gland materials cause precocious differentiation of unspecialized plant tissues?"

Experimental Methods and Results.

The observations here described were made entirely on roots growing from bulbs of *Narcissus*. Naturally, selection of a root must be made with reference to the special tissue structure it may exhibit. In some forms there is a clear-cut separation between the root-cap and its adjacent tissues; in such cases the cap may be cleanly removed from the adjoining tissues, there being no derivational dependence between them. In other roots the cap-cells are continuous with those of the root proper, a "common initial zone" existing between them, from which zone cells bud off into both adjoining areas. It is to this second type, "Type 5" of Haberlandt ('14), that *Narcissus* belongs; as a convenient reminder of the circumstances, a plan of its root tissue arrangement is shown in a diagram of its longitudinal section in Fig. 1. Neither the "common initial zone" nor the root-cap shows any distinct differentiation; they form an essentially parenchymatous mass. In a proximal direction, however, differentiation is soon met, *i.e.*, the cylindrical periblem, and the centrally placed plerome mass, also a cylinder.

The real question, then, which is being applied to the *Narcissus* root is: "Do the specialized tissues of the root proper extend down farther into the tip in such roots as are grown in nutritive media containing thyroid constituents than they do in roots grown in nutrient media alone?"

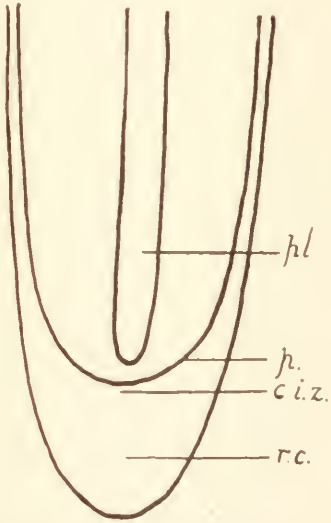

FIG. 1. Showing diagrammatically the arrangement of tissues in the root-tip of *Narcissus;* longitudinal section. *c.i.z.*, common initial zone; *p.*, periblem; *pl.*, plerome; *r.c.*, root cap.

Several dozens of *Narcissus* bulbs were rooted in bottles containing 120 cc. of Pfeffer's nutrient solution to which had been added gland substance (Parke Davis and Co.'s dry thyroid tablets) in these amounts: 2.5. grains, 5 grains, 7.5 grains, and 10 grains, respectively. It is well known, of course, that bulbs differ much in their "strength," and that in consequence of variation in vitality their root growth is not uniform in rate, length and other features; but there is an easily observable average which characterizes the normal or any experimental line. So far as bulk effect of thyroid substances on root growth in *Narcissus* goes, the result is the same as in *Allium*, reported by the writer in '19; in general, when the control bulbs showed a root length of 60 mm., the thyroid-"fed" line showed a length of 25 mm. These roots were then cut, fixed in aceto-alcohol, stained *in toto* in Delafield's hæmatoxylin, and embedded. Measurements of any region could be most accurately determined by a study of serial cross-sections; these were made 15 micra in thickness.

By count of sections, two measurements were made: (a) the interval from the tip of the root cap to the beginning of the periblem zone; (b) the interval from the tip of the root cap to the central plerome cylinder. To illustrate the uniformity of the result of such measurements, the following table is given, compiled from a series of thirty roots taken at random from among hundreds:

TABLE I.

MEASUREMENTS COMPUTED FROM SECTIONS, 15 MICRA IN THICKNESS, OF *Narcissus* ROOTS GROWING IN 120 CC. PFEFFER'S SOLUTION TO WHICH HAD BEEN ADDED THE EQUIVALENT OF 10 GRAINS OF THYROID GLAND.

	Micra from Tip of Root to Periblem.	Micra from Tip of Root to Plerome.
1	360	405
2	360	420
3	375	420
4	345	405
5	360	420
6	330	405
7	345	405
8	360	435
9	405	465
10	390	480
11	300	360
12	330	390
13	420	495
14	390	435
15	390	450
16	330	400
17	405	465
18	360	405
19	330	390
20	405	450
21	330	390
22	375	420
23	375	420
24	390	465
25	360	405
26	390	465
27	360	420
28	390	465
29	390	465
30	300	390

In the familiar experiments with frog tadpoles, the thyroid-fed line and the control line are, of course, taken from the same batch of eggs, thus being of the same nature and the same age. Similarly, in this case, the bulbs were sprouted in the same nutrient medium, for the same length of time, the sole difference between the experimental and the control bulbs being that the former grew with a given amount of thyroid material added to the solution.

For comparison with the above table, measurements similarly made of the control line are here added:

TABLE II.

MEASUREMENTS COMPUTED FROM SECTIONS, 15 MICRA IN THICKNESS, OF *Narcissus* ROOTS GROWING IN PFEFFER'S SOLUTION ALONE.

	Micra from Tip of Root to Periblem.	Micra from Tip of Root to Plerome.
1	450	555
2	465	550
3	540	630
4	480	540
5	420	480
6	450	495
7	480	555
8	450	525
9	465	540
10	480	540
11	465	525
12	480	540
13	435	495
14	465	510
15	420	495
16	435	495
17	450	510
18	495	555
19	450	510
20	435	495
21	495	570
22	420	465
23	495	570
24	480	540
25	495	570
26	495	570
27	495	555
28	405	480
29	450	525
30	495	570

It seems perfectly clear that the differentiated tissue extends nearer to the tip of the root in the thyroid treated line than it does in the roots of the control line: or, in tangible figures, it extends 20 per cent. nearer the tip.

DISCUSSION

The profound and almost semi-mysterious control of metabolism by thyroid constituents, expressing itself mainly in the phenomena of growth, has attracted many workers to an examination of very varied phases of its possible influence. The present study is but one of this sort; and certain aspects of the facts noted may well receive further comment.

As said above, the main point in mind has been to discover whether thyroid components, if absorbed by elementary rapidly growing plant tissues, will cause in them an accelerated differentiation comparable with what has been repeatedly described as a distinct effect of it when used as a food by, grafted into, or introduced as an extract into Amphibian larvæ. A primary awkwardness immediately presents itself in such an attempt, for the plant root here employed shows such limited specialization as compared with the complexity of an incipient animal organ such as an appendage. The periblem and plerome tissues are the only real differentiations from the parenchyma in a young root, and they show little character in their apical extremities, their identification being essentially dependent on position only. They are, however, *bona fide* differentiations, as truly as muscle, bone, or nerve tissue.

Furthermore, it would be folly to claim that the experimental results here reported are unique to, specific for thyroid "feeding"; there may be dozens of organic and inorganic compounds, so far as the writer knows, which could produce similar or even greater effects of the sort noted than do thyroid components. No attempt has been made to discover or compile such a list. Potassium biniodide has often been employed to offset the effect of thyroid deficiency in children. This fact, however, does not at all lessen one's interest in thyroid substances and their normal physiological values throughout the life-time of the possessing organism.

It may also seem that the interpretation given the experimental results of this study, as indicating precocious specialization, is arbitrary as against other possible judgments. For example, does not a long root of *Narcissus* normally possess a root-cap thicker than that of a short root? To test this point, a series of roots grown in nutrient solution alone were cut when at the same or less length than those of the thyroid "fed" line, the question of relative age being disregarded. On making measurements of these it was found that the short normal root shows an extent of root-cap equal to or even in excess of that of a longer normal root. The question here at issue can be most easily met by offering the following table of averages of root-cap length (tip to periblem and plerome) of thirty roots each, taken from long-normal (control), short-normal, and thyroid "fed" groups:

	Normal of Same Age as "Thyroid-fed."	Normal of Same or Less Length than "Thyroid-fed."	"Thyroid-fed."
Length of root up to beginning of periblem.	463 micra	513 micra	372 micra
Length of root up to beginning of plerome.	520.5 "	576 "	412.5 "

It is thus plainly apparent that the cap of the experimental line was more deeply invaded by the specialized tissue than were either of the check roots with which there was any reason for comparing it.

One also naturally raises the question whether the results noted, instead of being those of accelerated differentiation, could not as well be described as simple abbreviation or inhibition of root-cap growth. It is possible that such an interpretation would be perfectly valid; at the same time, one should reflect that the effect is one, not on the root-cap alone, but on the entire root. While the foregoing measurements give information as to only one feature, *i.e.*, length of root-cap, other specifications are also modified by the treatment: *e.g.*, its entire length is less than that of the normal; its average diameter is greater at nearly every part of its growth; it shows tendencies to localized swellings and crookedness. Measurements of these other features of the thyroid "fed" line, however, it is impossible to make in any satisfactory manner; yet, they may all indicate precociousness. Assuredly, one reflects that hastened specialization of tissues in amphibian larvæ, in dipteran larvæ, in protozoa, were all accompanied by under-size; and Gudernatsch's ('15) experiments with a mammal showed the same diminished size in his thyroid fed line.

The further question whether all protoplasm reacts, and reacts similarly to thyroid components can hardly be answered, even tentatively, while the amount of study of plant responses is so small. Also, one can feel only the most limited concern whether any special plant tissue is amenable to such hormones or not; but the more general inquiry as to the qualities of protoplasm as a whole makes a real appeal. So far as the substance of this group of observations goes, the evidence would seem to suggest an affirmative answer.

SUMMARY.

1. Roots growing from bulbs of *Narcissus* into Pfeffer's nutrient solution to which has been added certain amounts of thyroid gland substance show a growth which is markedly abbreviated as compared with that of the control lines. This result is wholly like that when *Allium* is used.

2. Measurements directed to find out the internal conditions correlated with shorter growth indicate that differentiation of the special root tissues extends nearer to the tip of the root in thyroid "fed" lines than it does in the controls.

3. Assuming that the tissues in freshly growing plant roots can be compared to those in larval animals (Amphibia)—both originate from essentially unspecialized masses, although the plant never reaches the complexity of the animal—it would seem that thyroid substances cause precocious differentiation in both.

4. The suggestion is offered that animal and plant protoplasm are sufficiently alike in their general physiology so that they respond similarly to thyroid substances; or, expressed conversely, thyroid substances are influential in essentially the same manner on all protoplasm.

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ON THE OCCURRENCE AND FOOD HABITS OF CTENOPHORES IN NEW JERSEY INLAND COASTAL WATERS.

THURLOW C. NELSON.

(From the Zoölogical Laboratory of Rutgers University and the Department of Biology of the New Jersey Experiment Station.)

I.

Studies of the plankton organisms of New Jersey inland coastal waters (Fig. 1) which have been in progress since March 1919 reveal interesting facts regarding the occurrence and habits of the ctenophores *Mnemiopsis leidyi* Agassiz, *Pleurobrachia brunnea* Mayer, and *Beroë ovata* Chamisso and Eisenhardt. All three of these forms have been found at times in abundance in New Jersey, but again they may be rare or absent. They must, therefore, be considered as transient visitors to these waters. This paper is presented with the hope that the observations herein recorded may aid in the ultimate solution of some of the problems concerned with the distribution of these comparatively little known animals.

The common comb-jelly or sea walnut of the northeastern coast of the United States, *Mnemiopsis leidyi*, was frequently found by Mayer ('12), off Newport, R. I., in great rafts. It was well known in the Woods Hole region until about 1910 when it practically disappeared and has not since been reported there in numbers.[1] Sumner, Osborn, and Cole ('13), p. 579, report this species as varying from scarce to very abundant throughout the Woods Hole region, where it has been recorded by various observers for every month in the year. It was apparently most abundant here in September and in December. These authors note the irregular occurrence of this ctenophore in different years, it being very abundant during some seasons while absent as in 1904 in others. They state that periods of extreme abundance may occur in winter. Sumner, *loc. cit.*, p. 576, records it in such numbers in Buzzards Bay on November 13, 1907, that the parasitic *Edwardsia leidyi* within the ctenophores were very conspicuous as one looked down from the deck of the ship.

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[1] Mr. George M. Gray, Curator of the Supply Department at Woods Hole, informs me in a letter of January 28, 1924, that nearly every year one or two specimens are taken in the winter or in spring. I have been informed that numerous *Mnemiopsis* were found here in September 1924 following an extended period of easterly winds.

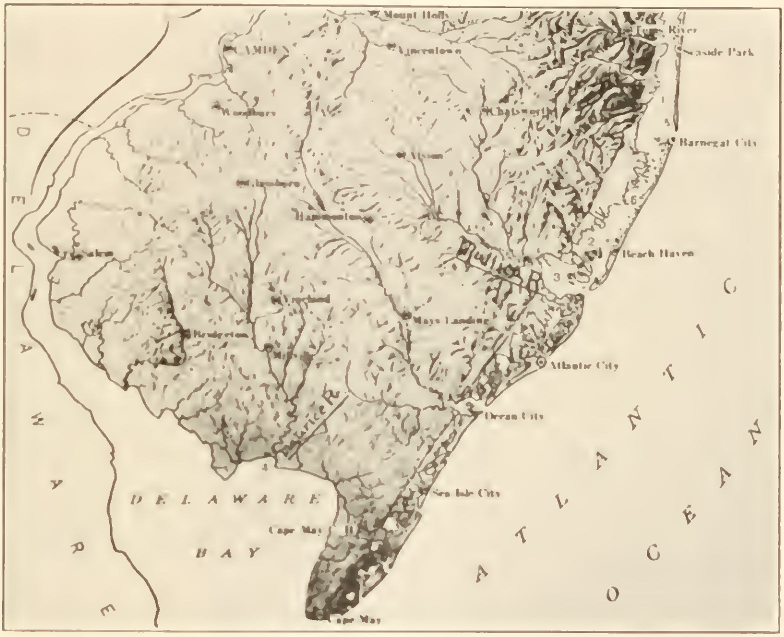
Mount Holly
Toms River
Seaside Park
Woodbury
Barnegat City
Hammonton
Beach Haven
Bridgeton
Mays Landing
Atlantic City
Ocean City
Sea Isle City
Cape May
1
2
3
4
6
DELAWARE
BAY

FIG. 1. Relief map of southern New Jersey. 1, Barnegat Bay; 2, Little Egg Harbor; 3, Great Bay; 4, Maurice River Cove. The floating laboratory was stationed from 1919–1920 at Edge Cove, due west of Beach Haven, and from 1921 to the present at Seaside Park.

Bigelow ('15) encountered "myriads" of *Mnemiopsis leidyi* in the surface waters off the New Jersey coast in July, 1913. He found it generally distributed over the inner half of the continental shelf between Barnegat and Delaware Bay, although none was seen north of Barnegat on this voyage.

Pleurobrachia brunnea nov. sp., was found in great numbers by Mayer ('12), on October 16, 1904, off the coast of New Jersey from Barnegat Inlet north to Sandy Hook. The validity of this form as a distinct species has been questioned by Bigelow ('12 and '15), and is discussed in a later section of this paper.

Mayer ('12) lists *Beroë ovata* as abundant along the coast of the United States as far north as Chesapeake Bay. Hargitt ('04) found it common at Woods Hole in 1901, though seldom taken in numbers.

My own records cover only some of the estuaries on the New Jersey coast and do not include the area of oceanic coastal water investigated by Mayer and by Bigelow. During the six years of my investigations *Pleurobrachia* has been found but twice, *Beroë ovata* for short periods during three seasons, while *Mnemiopsis* has been observed daily for months at a time.

Pleurobrachia brunnea was first found by me October 11, 1920, occurring in vast swarms at the surface of water 1–2 meters deep at the mouth of the Mullica River, Great Bay (*cf.* Fig. 1). Associated with it were numerous *Mnemiopsis leidyi* and many small medusæ of several species. So numerous were the *Pleurobrachia* that the water for yards around the boat was white as though with foam. The ctenophores were found in greatest abundance in the tidal slick which forms along the eastern end of the old Graveling natural oyster bed at the mouth of the Mullica River. The first observation of them was at 11:15 A.M., with bright sunlight, very light NW. wind, tide one third ebb, water temperature 16.6° C., specific gravity 1.0182.[2] The majority of the jellyfish were so close to the surface that they could be dipped up in a fingerbowl for examination.

During the afternoon of the same day large numbers of *Pleurobrachia* were found over much of Great Bay, and on October 15th, 6 *Pleurobrachia* and 3 *Mnemiopsis* were taken in 3 minutes towing with a 15-in. net in Little Egg Harbor. On my next visit to the region, three weeks later (November 3 and 4), no *Pleurobrachia* were found in Great Bay or in Little Egg Harbor. Numerous *Mnemiopsis* were obtained in the former region; the temperature was 13° C., specific gravity 1.0234.

Occasional visits have been made during the autumn in subsequent years but no *Pleurobrachia* have been found. From all the information I am able to gather this ctenophore appears in New Jersey estuaries for a brief period during October, and then disappears. Mr. Gray informs me that with one possible exception *Pleurobrachia* (supposedly *P. pileus*) was not seen in Woods Hole at all in 1923. That it did not occur in Great Bay during the summer of 1921 I am certain, since our floating laboratory was stationed during this period but a few hundred yards from the place where myriads of these ctenophores were observed in 1920.

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[2] All figures for specific gravity are reduced to the basis of distilled water at 4° C. Readings were made with an hydrometer calibrated by the U. S. Bureau of Standards and further checked in some instances by titration of standard sea water obtained from the International Commission for the Investigation of the Sea, Copenhagen. For comparison I have with the aid of Knudsen's Tables transposed Bigelow's salinity figures to specific gravity readings.

My first record for *Beroë* is November 4, 1920, when one half grown specimen was taken in Great Bay; the water temperature was 13° C., specific gravity, 1.0216. No further specimens were seen until October 22, 1921, when 2 large individuals were observed in Little Egg Harbor with temperature of 12.5° C., specific gravity 1.0245. No *Beroë* were found in Little Egg Harbor during the autumn of 1922, nor in Barnegat Bay in the autumn of 1923. A few large specimens were taken in the latter region September 11, 1924, water temperature 16.5° C., and by the close of the month they were abundant. This ctenophore appeared in vast swarms, in the Maurice River, Delaware Bay, about the middle of September 1922 and again at the same time in 1923 and in 1924.[3] It was reported that at times during the flood tide the *Beroë* were so abundant as almost to form continuous rafts. In each of these years the ctenophores appeared about the second week in September while the water temperature was above 20° C. and remained very abundant until early in October when they disappeared as quickly as they had come.

My records of *Mnemiopsis* are much more extensive than in the case of the two preceding ctenophores. The data illustrate in a striking way what other investigators have noted regarding the intermittent appearance of the sea walnut. During the summers from 1908 to 1917 I frequently noted the presence of *Mnemiopsis*, but being occupied with other problems kept no record of its occurrence. From early in 1919 to the present careful records have been kept of the appearance and relative abundance of the ctenophores. Search for these organisms has

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[3] I am indebted to two of our former students, Mr. C. A. Perry and Mr. W. H. Dumont, for making these observations and for sending specimens for identification.

TABLE I.

OCCURRENCE OF *Mnemiopsis leidyi* IN NEW JERSEY.

Locality	1919	1920	1921	1922	1923	1924
Little Egg Harbor	Very abundant May to November, 2 qts. obtained in 20 min. tide flow with 15 in. net.	Very scarce during summer. Three taken in 5 min. tide flow on Nov. 4th, temp. 10.6° C. None Nov. 19, temp. 6° C.	None in net in 2 hrs. tide flow May and September. Two caught in a Urosalpinx trap.	None found.	None May 7th; Fairly abundant in late August, remainder of season not known.	Few seen July 24–25. Remainder of season not known.
Great Bay	Not known.	Scarce during summer. Numerous on Oct. 11th with Pleurobrachia. Eight taken in 2 min. tide flow November 3.	None found.	None found.	Occasional specimen seen in late August, Remainder of season not known.	Numerous July 24th. Remainder of season not known.
Barnegat Bay	Not known.	Not known.	Scarce. One specimen 10 cm. long taken in lower Bay Aug. 31. Hit 1 about every 300 ft. at night with launch.	None found. Hit 1 occasionally at night.	Appeared suddenly June 21, specimens of medium size. Increased rapidly and occurred in swarms throughout summer and fall. Very abundant in December, scarce in January, disappeared during February.	Six specimens seen July 3. Great swarms July 8–18.

been made by nets, by traps, and, perhaps best of all where they are scarce, by running a motor boat at night and counting the number of individuals flashing in the wake of the boat. Much of the region covered by these observations is so shallow that the disturbance from a launch's propellor reaches quite to the bottom.

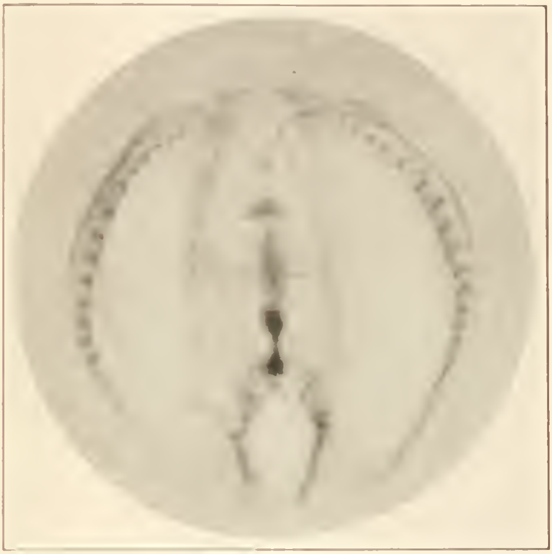

FIG. 2. Young *Mnemiopsis* 5 mm. long in the *Pleurobrachia* stage showing retracted tentacles.

In Table I. a summary is given of our observations of *Mnemiopsis* for the past six years.

Barnegat Bay (*cf.* Nelson, '23.1) is a shallow estuary with an average tidal fluctuation of 4-6 inches. It therefore warms up rapidly in the spring and presents during the summer subtropical temperature conditions. The temperature of the water on June 21, the date of the first appearance of *Mnemiopsis* here in 1923, ranged from 24.5° to 26.1° C. at 7 stations. The specific gravity varied between 1.0117 and 1.0152. The ctenophores which swarmed in the waters of the bay during July, August and September were mostly of medium or of large size.

As the temperature fell with early autumn particular attention was paid to the stage of development of the animals. All specimens taken October 6, at a temperature of 14° C., were medium or large. On my next visit, October 20, temperature 15° C., I found several great rafts of *Mnemiopsis* about two thirds of the individuals being approximately 5 mm. long. Among these there were both *Pleurobrachia* and *Bolinopsis* stages of development, bearing strong tentacles (Fig. 2). The smallest were not more than a millimeter long. As indicating the abundance of the animals, one 450 cc. fingerbowl dipped up full of the surface water contained in all 36 young *Mnemiopsis* of which 24 were in the *Pleurobrachia* stage and 12 in the *Bolinopsis* stage. The majority of specimens found on subsequent visits during November, December and January were in the *Pleurobrachia* stage, relatively few large specimens being taken.

In the summer of 1924 the first specimens, 6 of medium size, were found July 3, water temperatures 21.5–22.7° C. Few were observed after this until the 8th when, with temperatures of 24.5 to 24.9° C, the water was literally alive with *Mnemiopsis*. Most of these were newly hatched embryos corresponding to Mayer's Fig. 35 (Mayer, '12, Pl. 6) which represents an embryo of about 30 hours.

Following this heavy spawning of July 6–8 the water swarmed with the ctenophores, numbers as high as 100 per cubic meter being recorded. The animals continued abundant for about 10 days after which they diminished rapidly in numbers, and at no time after this did they approximate the swarms seen in 1923.

The lower limit of temperature at which *M. leidyi* will breed must be very close to freezing, as the following observations show. The fall of 1923 and January 1924 were very mild; our self-registering water thermograph on the Maurice River, Delaware Bay, rarely fell below 5° C. until late in December. The first freeze in Barnegat Bay occurred on the night of January 5th, the bay being covered with ice for about three days. On the morning of January 5, with a temperature of 2° C., numerous medium and small Mnemiopsis were procured.[4]

On my next visit to the region, January 19, no specimens were taken in repeated hauls in the open bay. Medium and very small individuals were fairly abundant, however, in two artificial harbors about 2 meters deep which communicate with the bay by narrow outlets. Although these harbors had been frozen over from the 5th of the month until about a week before my visit, the quietness and depth of the water had apparently provided conditions more favorable to survival of the ctenophores than obtained in the open bay. In horizontal hauls of 8 meters each with a net 1 meter square, as high as 19 *Mnemiopsis* were procured per haul. Of these approximately 90 per cent. were in the *Pleurobrachia* and *Bolinopsis* stages and must have come from eggs liberated since the cold weather early in the month.

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[4] I am indebted to Mr. Wible of the Physiology Department of Rutgers University for this observation.

No further visits were made to the region until March, since the harbors and much of the bay were ice bound from late January until the close of February. On March 8, 1924, water temperature, 3.5° C., no *Mnemiopsis* were found. The adverse conditions resulting from the heavy freezing had evidently destroyed the few survivors in the harbors where they were found on January 19th. The disappearance of *Mnemiopsis* from Little Egg Harbor in November and from the open waters of Barnegat Bay early in January is believed to be due chiefly to the effects of heavy storms which raise much sand and debris from the bottom.

III.

The Behavior of *Mnemiopsis* at Low Temperatures.

Parker '05 found while working with *M. leidyi* during the summer at Woods Hole, that partial cessation of the paddle plates occurred by chilling to 8.5° C., with complete loss of movement at 5° C. The absence of movement at this temperature was shown to be due to causes other than the failure of nervous transmission. If 5° C. represented the temperature below which, in their natural environment, movement of the paddle plates of *Mnemiopsis* ceased, the organisms would perish before late autumn in New Jersey. The temperature of 5° C. as determined by Parker must represent a minimum only for *Mnemiopsis* adjusted to summer temperatures.

At sunset November 9 with the water temperature of Barnegat Bay at 7.5° C., large numbers of *Mnemiopsis* of all sizes were found wherever sought. The following night was cold with a sharp north wind. At 8:30 the next morning numerous *Mnemiopsis* were seen swimming actively at the surface near the floating laboratory in water with a temperature of 3° C. Several animals were at once dipped up to make certain that the swimming plates were actually in motion.

To determine the minimum temperature at which the paddle plates would beat in winter, on January 19, 1924, I took *Mnemiopsis* from the water beside the laboratory at a temperature of 4° C. With the aid of a pack of ice and salt the water containing the animals was cooled down until at—0.7° C. water and ctenophores became a mass of ice.[5] As the temperature fell the paddle plates continued beating without interruption. Ice crystals formed about the ctenophores, finally enclosing them, yet while half of an animal was solidly embedded in the advancing ice the paddle plates of the free half continued beating as before. Not until the impinging ice crystals actually imprisoned the plates and held them fast did movement cease.

Mayer ('14) emphasized the fact that whereas tropical marine animals commonly live within 5° C. of their temperature of maximum activity and within 10–15° C. of their upper death temperatures, marine animals of the temperate or arctic regions show but little change in activity within a considerable range of temperatures. Hunter ('04) showed that *Mnemiopsis leidyi* is relatively more resistant to a decrease than to an increase in temperature of the water. The bearing of these observations on the distribution of *Mnemiopsis* in New Jersey will be considered in the last section of this paper.

IV.

THE FOOD HABITS OF CTENOPHORES.

Little is known of the feeding and food habits of ctenophores. Their delicate structure and relatively short life under laboratory conditions, together with their somewhat sporadic appearance within the reach of laboratories, have made investigation of their habits difficult. Mayer ('12) notes that "young *Beroë cucumis*" devours *Pleurobrachia* "with avidity." Bigelow ('15) observed the great impoverishment of the plankton on German Bank due to *Pleurobrachia pileus*, which "when it swarms seems to obliterate or devour almost everything else in the water." Kincaid ('15) states that *Pleurobrachia* may be a serious enemy of the oyster in Washington waters through the large numbers of the larvæ of the latter which it consumes.

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[5] Moore ('24) observed the beating of the paddle plates of *Mnemiopsis* at − 0.6° C. under laboratory conditions.

In a recent work, the most extensive of its kind with which I am familiar, Miss Lebour ('22, '23) describes the food of numerous plankton organisms. She lists *Pleurobrachia* and *Beroë* with *Sagitta* among the miscellaneous feeders of the plankton. Where these two ctenophores occur together *Beroë* may eat large numbers of *Pleurobrachia*, an observation which was also published by Mayer, however, in 1912. The chief food of *Beroë* was found by Miss Lebour to be small crustacea, although she quotes one observation of a *Beroë* full of diatoms, probably *Coscinodiscus*. *Pleurobrachia* was found to subsist mainly upon *Calanus*, crab zœa larvæ, *Sagitta*, other *Pleurobrachia*, *Syngnathus*, young plaice and plaice eggs. I have not had opportunity to determine the food of *Pleurobrachia*. The *Beroë* which were found September 11, 1924, were living chiefly upon *Mnemiopsis*.

The abundance and persistence of *Mnemiopsis* in Barnegat Bay in 1923 gave opportunity to study the food habits of this ctenophore during the seasonal changes of the plankton. A few preliminary examinations demonstrated that at summer temperatures the rate of digestion of food and the ejection of residue is so rapid as to make necessary the examination of the ctenophores immediately upon removal from the water. In most instances the stomodeum was emptied of all its contents in from 20–30 minutes after removal of the animals from their natural surroundings.

The great transparency of this organism makes it possible to identify with a high degree of accuracy the contents of the stomodeum without in any way disturbing the animal. In making the examinations the station launch was anchored in the desired spot in the open bay and the ctenophores were dipped up in a fingerbowl as needed and examined immediately under the binocular.

During the summer the food of *Mnemiopsis* was found to consist chiefly of larval molluscs, copepods and their nauplii, nannoplankton, and detritus, the relative amounts of these being to some extent correlated with their abundance in the plankton. In this connection I wish to lay emphasis upon a fact which, so far as my knowledge goes, has not hitherto been stressed. The presence of nannoplankton and of organic debris within the ctenophores demonstrates that these animals make use of their ciliated canals for the transport of minute forms in much the same manner as does a bivalve mollusc. The possession of this feeding mechanism makes available to them the nannoplankton which is by far the greatest constituent of the total plankton, a constituent, moreover, which may be mainly unavailable to their larger cœlenterate allies. This may explain, in part, how such vast hordes of ctenophores can exist together for long periods of time (*cf.* Nelson, '22).

Table II. contains a summary of the food organisms found in *Mnemiopsis* during July.

To summarize the data in Table II.: of 65 *Mnemiopsis* examined during July, 75 per cent. had eaten bivalve larvæ, 50 per cent. contained crustacea, 15 per cent. held gastropod larvæ, while 6 per cent. contained detritus and nannoplankton. One specimen about 3 cm. long had eaten 126 early oyster larvæ.

On December 21, of 10 specimens examined 5 contained a total of 26 large *Calanus* and 1 gastropod larva. Examination of the ctenophores at this time and again on January 19, 1924, revealed a most interesting fact regarding digestion in *Mnemiopsis*. Copepods, as is well known, contain large oil globules, representing stored nutriment. In every ctenophore examined during the winter, oil globules derived from the copepods, were found to be deposited in thick rows beneath the paddle plates. Many minute oil globules were seen passing out of the anus, and one specimen was observed in the act of casting out through the mouth a thick rope of oil globules and detritus. Apparently but little of the oil obtained from the crustaceans used as food is metabolized by the ctenophores, at least at low temperatures. The storage of the oil in such large quantities beneath the paddle plates may serve an important function in decreasing the specific gravity of the body. This accumulation of oil at a time when also the density and the viscosity of the water are greatly increased through low temperature, renders the animal capable of suspension in the water with a minimum of activity of the paddle plates. That the added factor of buoyancy has survival value during the late fall and winter can scarcely be doubted.

TABLE II.

Date.	Total Number Examined.	Oyster Larvæ.		Other Bivalve Larvæ.		Crustacea.		Gastropod Larvæ.		Detritus.		Miscellaneous.
		Larvæ.	*Mnemiopsis.*	Larvæ.	*Mnemiopsis.*	Copepods and *Nauplii.*	*Mnemiopsis.*	Larvæ.	*Mnemiopsis.*	Amount.	*Mnemiopsis.*	
July 12....	11	301	9	2	2	1	1	3	1			
July 13....	40	74	12	58	14	93	35	3	2	Much	2	1 roundworm 1 atax
July 18 ...	14	77	12			21	6	12	7	Much	2	1 prawn, 3 mm.
Total....	65	452	33	60	16	115	42	18	10		4	

An interesting observation illustrates one limitation of *Mnemiopsis* in its ability to capture planktonts. The water was swarming with polychæte larvæ on December 21, but in spite of the fact that many of these larvæ were found entangled in mucus on the oral lobes and on other parts of the bodies of the ctenophores, in no case were any larvæ found within the stomodeum. The wealth of spines which bristle in all directions on the early larvæ of polychætes evidently render their final capture by *Mnemiopsis* difficult if not impossible.

That the food organisms found within the stomodeum of *Mnemiopsis* are actually digested is shown by the presence of numerous empty shells of bivalve and gastropod larvæ, crustacean carapaces, and other non-digestible remains which accumulate near the oral end of the stomodeum.

V.

THE DESTRUCTION OF BIVALVE LARVÆ BY *Mnemiopsis*.

Our records of the abundance of bivalve larvæ and the intensity of "set" in Barnegat Bay for the past 3 years show, after taking into account the effects of all known factors, that there is a close correlation between the abundance of *Mnemiopsis* and the intensity of shipworm infestation and of oyster sets. In 1921 and in 1922 heavy sets of *Ostrea*, *Teredo*, and *Bankia* occurred. The oyster set in 1921, especially, was the heaviest seen for some years, as high as 1000 oyster spat attaching to one oyster shell (Nelson, '23A). The sudden and heavy outbreak of *Teredo navalis* at the same time (Nelson, '22) gave rise to fears that there might be enacted in eastern waters a repetition of the San Francisco Bay disaster (Kofoid et al., '21). It will be noted in Table I. that *Mnemiopsis* was absent or rare in Barnegat Bay and adjoining waters during 1921 and 1922.

The oyster set in Barnegat Bay in 1923 was a failure commercially. The best set that could be found at the close of the summer was one or two spat on every third oyster shell. The total season's catch on certain experimental shells was only 7 spat as against over 7000 in 1921.

Teredo navalis infested timbers in numbers as great as 100 per cubic inch of wood in 1921, while in 1923 at the same spot only 10 *Bankia* entered a test raft of 1,520 square inches surface. This very light infestation occurred in spite of the prevalence of slightly higher salinities obtaining in the region than were found in 1921. At only one locality, the jetty at the mouth of Barnegat Creek at the lower end of the Bay, could enough *Teredo* be found even for carrying on experiments with them. This jetty has been heavily attacked by borers for some years and was practically destroyed in 1921. Two infested piling were removed from this structure on July 25 and 5 were taken August 31, 1923. They were moored in a small land-locked creek close to the upper end of the bay, far from marine structures, and were used as a source of supply for study of the heterotrichous ciliate *Boveria teredinidi* Nelson, parasitic upon the gill filaments (Nelson, '23*B*). Throughout the summer and autumn until freezing occurred the borers grew and flourished, thus proving that no natural conditions present in the bay during this period were inimical to the existence of the adults.

The Director of the Committee on Marine Piling Investigations of the National Research Council, Col. Wm. G. Atwood, informed me in a letter of October 23, 1923, that "on the whole shipworm attack on test blocks was much lighter during 1923 than in 1922, although at one or two points it appears to have been as heavy or heavier." Unfortunately for the purposes of this discussion there are no data as to the relative abundance of *Mnemiopsis* or of other plankton feeders in the several regions.

The relative reduction in numbers of oyster larvæ in 1921 and in 1923 is of interest. For example, on June 25, 1921, the average number of earliest straight hinge oyster larvæ per 100 liters of water, collected at 7 stations on Barnegat Bay, was 36,200. On the 27th, by which time the larvæ were entering on the early umbo stage, the average number of these larvæ was found to have been reduced to 8,700, representing a quite usual precentage of mortality. In 1923, on June 23, at the central collecting station near the middle of the bay where the early oyster larvæ have been first found in abundance for three successive years, there was obtained a total of 60,850 larvæ per 100 liters. Two days later the average of 8 stations showed but 54 of these larvæ remaining per 100 liters, the largest single catch at any one station being 200. So far as known there were present during 1923 no other factors aside from vast swarms of *Mnemiopsis* which were not also operating in 1921.

It is worth while to add a comparison between oyster sets found in Barnegat Bay by late August, 1923, with those at other oyster producing areas of New Jersey. In the Mullica River an excellent set of oysters one week old was found on August 30. At this date only an occasional *Mnemiopsis* was seen. Although no observations were made in this area earlier in the season there is no reason for believing that the ctenophores were more abundant during the preceding 3 weeks, during which time the oyster larvæ would have matured and set, than they were on the date of my observations. In Delaware Bay also the usual oyster set occurred. The waters of this area are so stormy and turbid near the shore that *Mnemiopsis* cannot thrive there, although the much hardier *Beroë* appears there in abundance in September, as already stated.

VI.

Discussion of Species.

To the student of the Ctenophora it is apparent that certain wide discrepancies occur between the account of the distribution of the three ctenophores discussed in this paper and the commonly accepted ideas regarding the habitat of these species. All are usually considered as being strictly oceanic forms, and yet I have taken them in waters ranging from one half to less than a third the salinity of their supposed usual environment.

Mayer '12 holds that there are but two dominant species of *Beroë*, *B. cucumis* of cold waters and *B. ovata* of warm water. *B. forskali*, which was found at numerous stations by Bigelow, is believed by Mayer to be but a variety of *B. ovata*. Bigelow ('15) lists *B. cucumis* from 7 stations at some distance from the coast of New England. He does not record it off the New Jersey coast in July, nor does he mention *B. ovata*.

Mayer ('12) notes that the young of *B. cucumis* cannot be distinguished from those of *B. ovata*. In the adult the peripheral network of vessels arising from the meridional canals freely anastomoses in *B. ovata*, remaining distinct in *B. cucumis*.

Pleurobrachia brunnea, a species created by Mayer '12 to receive a ctenophore found off the New Jersey coast in October, 1904, is of somewhat doubtful position. Bigelow ('12) believed this to be so close to *Hormiphora spatulata* Chun, as to be identical with it. In a later publication ('15) he throws still further doubt on the validity of the species, believing it to be well within the limits of *P. pileus*, a ctenophore of wide distribution which he found in great numbers off the New Jersey coast in July 1913.

The characters used by Mayer to establish *P. brunnea* as distinct from *P. pileus* are: the continuation of the 8 meridional canals for a considerable distance downward beyond the ciliary combs; the yellow color of the stomodeum; and the knobs on the ends of the tentacles. It is distinguished from *Hormiphora spatulata* by the knob-like ends of the tentacles; and by the fact that its ciliated combs begin at a greater distance from the apex than in *H. spatulata*.

My own specimens agree in every particular with Mayer's description and figure of *P. brunnea*, the yellow color of the stomodeum and the knob-like ends of the tentacles being quite striking in the living specimens. The downward continuation of the meridional vessels below the ciliary combs is clearly evident in the preserved as well as in the living specimens. In considering Bigelow's belief that this form may lie within the limits of variation of typical *P. pileus* it is well to remember that

Bigelow obtained his specimens off the New Jersey coast in July, whereas Mayer and I found our ctenophores in October. The three distinguishing characters of *P. brunnea* as established by Mayer are clearly evident even to one who is but little familiar with the characteristics of this group. The chief question would seem to be whether this organism should be raised to specific rank, or be included as a variety of *P. pileus* or of *Hormiphora spatulata*. Its appearance in New Jersey coastal waters in October would seem to point to its being essentially a cool water form and hence closer to *P. pileus*.

The range of *Mnemiopsis leidyi* is given by Mayer as from the southern coast of New England south to the Carolinas. It is considered by Mayer to be a ctenophore of the pure sea water along the outer shores; its place being taken in the brackish water by its much smaller relative, *M. gardeni*. Bigelow ('15) agrees with this from his own findings. Nowhere in the accounts of either of these investigators do I find evidence that they made any careful examination of the estuaries of the New Jersey coast for there *M. leidyi* occurs in abundance for months at a time in water of as low as one-third the salinity of the sea or even below this. On one occasion I found it in upper Delaware Bay, just below Stony Point, in water of a specific gravity less than 1.005.

That I have not confused *M. leidyi* with *M. gardeni* will be evident from the following: (1) *M. gardeni* is described by Mayer as being 35–40 mm. in length when mature. I have found *Mnemiopsis* over 100 mm. long in Barnegat Bay. (2) The oral lobes of *M. gardeni* are very small, from one-fifth to one-sixth as long as the body. In all specimens of *Mnemiopsis* which I have seen, excepting only immature individuals not yet fully emerged into the Mnemiopsis stage, the oral lappets are much longer than this and they flare widely. (3) My specimens show, when large numbers are together, a decided pinkish hue, not the bluish color of *M. gardeni*. (4) The range for *M. gardeni* as given by Mayer is from Chesapeake Bay to Florida. (5) the striking power of adaptation of our species to low temperatures argues against its being the southern form. (6) I do not find in my specimens the small low discoidal warts on the oral lobes which according to Mayer are characteristic of *M. gardeni*.

VII.

The Distribution of Ctenophores as Effected by Salinity and Temperature.

Mayer ('14) has emphasized the superior temperature adjustment of marine animals of the arctic and temperate zones over individuals of the same or of related species in the tropics. *Mnemiopsis leidyi* in Barnegat Bay illustrates this superiority of adjustment in a striking manner. Owing to the typography and small tidal flow of Barnegat Bay, summer temperatures there may rise well above 26° C. During most of the summer of 1923 the water temperature varied between 24 and 25° C., with specific gravity over the region within 4 miles south of Seaside Park ranging from about 1.0110 to 1.0180.

Bigelow ('15) found *Mnemiopsis leidyi* in July in greatest abundance over the inner half of the continental shelf off the New Jersey coast, in water of a specific gravity between 1.0246 and 1.0252. It was not found in the very salt Gulf stream on the one hand nor where the specific gravity fell below 1.0244 at the mouth of Chesapeake Bay, on the other. The upper limit of temperature at which this ctenophore was found was 24.5° C., the lower about 15.5° C.

He gives the range of *Pleurobrachia pileus* as unbroken from Labrador at least to Pamlico Sound. He found it more generally distributed in the coast waters than any other cœlenterate, with local swarms south as well as north of Cape Cod. The warmest water in which it was found was 20.5° C., the coldest about 6° C. The range in specific gravity was from about 1.0241 to approximately 1.0267. Nearly all the specimens taken were from the deeper waters, whereas the majority of *Mnemiopsis* were found in the first fathom. The swarms of *Mnemiopsis* and *Pleurobrachia* were found to be mutually exclusive; never were these two forms taken side by side.

The distribution of *Pleurobrachia* in the deeper waters, together with the lower temperatures observed, indicate that *Pleurobrachia* is adjusted to colder water off the New Jersey coast than is *Mnemiopsis*. The high summer temperatures of the estuaries such as Barnegat Bay form a barrier which it, unlike *Mnemiopsis*, is unable to pass. In the extreme southern part of its range

Pleurobrachia is undoubtedly adjusted to temperatures equal to if not above those found during the summer in Barnegat Bay.

Pleurobrachia brunnea may be a species or subspecies which is delicately adjusted to a narrow range of temperature, since it is found in the inland waterways of New Jersey only during October, and it was taken in the ocean off Barnegat by Mayer during the same month. It is not stated at what depth these were obtained nor is the temperature given. As bearing on Bigelow's finding that *Pleurobrachia pileus* and *Mnemiopsis* were mutually exclusive, it will be remembered that in the swarms observed by me in the Mullica River *P. brunnea* and *M. leidyi* were taken together in large numbers at the surface in water dipped up with a fingerbowl.

In conclusion, consideration must be given to the part which salinity and temperature play in the sporadic appearance of the three ctenophores discussed here. A study of all data relating to rainfall, seasonal temperatures, severity of the preceding winter, and other factors, fails to show any correlation between these and the abundance of the ctenophores. The fact that one or two specimens of *Mnemiopsis* have been taken every year at Woods Hole since they ceased to be found there in abundance, and the presence of occasional specimens in Barnegat Bay even during "off" years, indicates that the factors controlling the appearance of the ctenophores probably lie mainly outside the several localities which are considered in this paper.

All ctenophores disappear from the shallow bays during the winter, the waters being repopulated the following summer from the sea. Should the region in which Bigelow found *Mnemiopsis* in such abundance in the Atlantic Ocean off the New Jersey coast be shifted even a mile or two eastward during some years, it would probably pass beyond the influence of the tidal ebb and flow through Barnegat Inlet and as a result few if any *Mnemiopsis* would be found in the Bay. Long continued easterly winds do bring to our shores forms which do not normally occur there but the effects of such winds and the currents produced thereby are doubtless only temporary.

No one knows the effects on the plankton of coastal waters which result from even slight shifts in the direction, and of changes in temperature or of salinity of the larger oceanic currents. Until extensive oceanographic investigations are conducted along our coasts during every month of the year for a considerable period, we shall continue ignorant of most of the underlying factors which finally determine the abundance and distribution of many of the transient visitors to our coastal waters.

SUMMARY.

The occurrence and distribution of the three ctenophores, *Mnemiopsis leidyi*, *Pleurobrachia brunnea*, and *Beroë ovata* in New Jersey inland coastal waters are considered as affected by factors of the environment.

Pleurobrachia was observed twice, *Beroë* during a few days for three seasons, *Mnemiopsis* was seen daily for months at a time.

Evidence is presented which indicates that all three of these forms are casual visitors to the inland coastal waterways of New Jersey. *Beroë* and *Pleurobrachia* are found here only after the heat of summer is past, whereas *Mnemiopsis* appears in late spring or early summer and flourishes until late autumn or winter.

The remarkable power of temperature adaptation of *Mnemiopsis* is shown by the fact that in individuals taken in the winter the swimming plates continue to beat until frozen fast in the ice.

The three ctenophores are shown to be able to withstand salinities in the bays which are considerably below those of the ocean lanes from which the animals came. The conclusions of Mayer and of Bigelow in this connection are discussed.

A study of the food habits of Mnemiopsis reveals the fact that large numbers of bivalve larvæ may be destroyed by them. A distinct correlation is shown between the abundance of *Mnemiopsis* and the intensity of "sets" of oysters and of marine borers.

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STUDIES ON MICROSPORIDIA PARASITIC IN MOSQUITOES.

V. Further Observations upon *Stempellia* (*Thelohania*) *magna* Kudo, Parasitic in *Culex pipiens* and *C. territans*.[1]

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Introduction.

In the autumn of 1919, I chanced to observe at Urbana, Illinois, six larvæ of *Culex pipiens* which were infected by a microsporidian and named it *Thelohania magna* (Kudo, '20). Since the prospect of obtaining additional material was small at that time, I ventured to summarize the results of my observations made upon "a single section preparation and a number of smears" of the host larvæ in a paper ('21).

In the following year, I found at Warren, Pennsylvania, some forty-three larvæ of *Culex territans* which harbored an apparently similar protozoön, whose effect upon the host mosquitoes was the subject of another paper ('22). This material from Pennsylvania was far more abundant than the previous collection, and enabled me to conduct an infection experiment. The observations in the temporary field laboratory and subsequent microscopical studies of a large number of preparations have brought into light some phases of the development of the protozoön which were not recognized in the scant material of 1919 and which now lead me to place the microsporidian in the genus *Stempellia.*

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[1] Contributions from the Zoölogical Laboratory of the University of Illinois, No. 254.

The present paper deals with the findings thus made with special reference to the development of the microsporidian starting with an experimental infection and is intended to supplement the observations published before.

MATERIAL AND METHODS.

The host larvæ, *Culex territans*, were found breeding in an old boat filled with rain water on Conwango River at Warren, Pennsylvania. On two occasions, July 5 and 10, 1920, 290 larvæ were collected and examined microscopically, of which 43 were found to be infected by the microsporidian.

The material was studied in fresh as well as fixed and stained smears and in section preparations. For fixation, Schaudinn's mixture was mainly used; for staining, Heidenhain's iron hæmatoxylin or Giemsa's stain was used as in previous studies. A few Giemsa-stained preparations were decolorized and stained with Heidenhain's hæmatoxylin, although the reverse was not attempted, in order to compare the effects of staining by the two methods. It may be worth while to state here that methylene blue M.P. seems to be a suitable stain for the spores. On July 7, 1920, I subjected a number of spores to mechanical pressure on a slide, added one drop of a strong aqueous solution of methylene blue and sealed with a vaselined coverglass. An examination of this preparation in the summer of 1923 showed that the spore membrane had not shrunk and that both the polar capsule and the sporoplasm in the spores which escaped the pressure were most distinctly visible (Figs. 4 *j, k*).

Since Fontana was not carried on the trip, smears of pressed spores were either stained with Giemsa's stain or treated with a mixture of Lugol's solution and gum arabic. Some of the smears were kept air-dry, however, and stained later with Fontana.

THE INFECTION EXPERIMENT.

In order to determine the changes which the spores of the microsporidian undergo in the digestive tract of a new host larva when taken into it with food and if possible, the way with which the parasite becomes established in the new host animal, an experiment was conducted.

A number of larvæ of *Culex territans* which had been collected on July 10 and which were normal in appearance and behavior, were set aside in a glass jar. At two o'clock in the afternoon, three heavily infected larvæ, cut into small pieces, were given the larvæ, which fed willingly upon these fragments immediately after the latter were placed in the water. Some of the larvæ fed only for a few seconds, others for several minutes. The latter were removed one by one by means of a pipette as soon as they ceased to feed, into another jar which was partly filled with the rain water that had been standing by the laboratory free from mosquito larvæ. Twenty fed larvæ were thus obtained by three o'clock. I was sure that they had eaten certain portions of the infected material; in fact, all of them showed, upon microscopical examinations which followed in from six hours to four days, that they had devoured a large number of spores and sporulating stages of the parasite. The majority of the larvæ which did not feed long or at all, were found pupated on the following days.

From another lot twenty larvæ were selected as control animals by macroscopical inspection of the group and a low power microscopical examination of the individual larvæ.

On account of the comparatively simple organization of the alimentary canal and its connected organs of the mosquito larva and of the large dimensions of the protozoön, the microscopical examinations of the material involved in the experiment both in smears and in sections were carried out with greater ease and certainty than that which I had experienced in the case of the infection experiments of *Bombyx mori* with *Nosema bombycis* (Kudo, '16), where the conditions were reversed.

In the study of the preparations related to the experimental infection, Giemsa's stain seemed to be indispensable, since it brought out the spores, particularly the sporoplasms with their nuclei, of the microsporidian, sharply before the multitude of other microorganisms of animal as well as plant nature, which existed in the rain water and which found their way freely into the alimentary canal of the insects. The distinction between the latter and the emerged sporoplasms of the present microsporidian was the matter which occupied a great deal of my time. It was found that this distinction could only be done with great difficulty in the preparations stained with Heidenhain's iron hæmatoxylin, while it was comparatively easily made out in the sections stained with Giemsa's mixture. With Giemsa, the nucleus of a sporoplasm whether within the spore membrane or without, took a peculiarly bright crimson color, which led me to distinguish it possibly as such from those of the other organisms present in the lumen of the host gut.

The scheme of examination of both the experiment and the control larvæ was as follows:[2]

Time after Feeding on Infected Material.	Observations in	
	Smears.	Sections.
6 hours. .	The alimentary canal of two larvæ was extracted from the body and studied in fresh conditions; later fixed and stained.	Two larvæ were fixed *in toto*.
24 hours. .	Two larvæ observed in a way similar to the above.	Two larvæ were fixed *in toto*.
40 hours. .	Four larvæ observed in a way similar to the above.	
2 days. .	The entire body of two larvæ was studied in fresh smears; later fixed and stained.	Two larvæ were fixed *in toto*.
4 days. .	. . . .	Four larvæ were fixed *in toto*.

In the fresh preparations of the alimentary canal of the larvæ which were examined six hours after feeding on the infected material, a large number of unchanged spores with normal appearances were observed. Spores with a comparatively large clear area at the rounded extremity were noted in numbers (Fig. 1, *a*); spores with a small mass projecting from the attenuated end were also noticed (Fig. 1, *a*). When stained, the spores showed a relatively large nucleus (Fig. 1, *b*).

In the larvæ which were examined 24 hours after feeding on the infected material, the mid-gut contained a large number of unchanged spores and empty spores; some of the latter showed extruded filaments. A number of spores exhibited an appearance shown in Fig. 1, *c* in which apparently the emergence of the contents was taking place. This particular spore sketched here was kept under observation for thirty minutes with an oil

[2] Due to unexpected early departure from the place, the experiment was unfortunately discontinued on July 14.

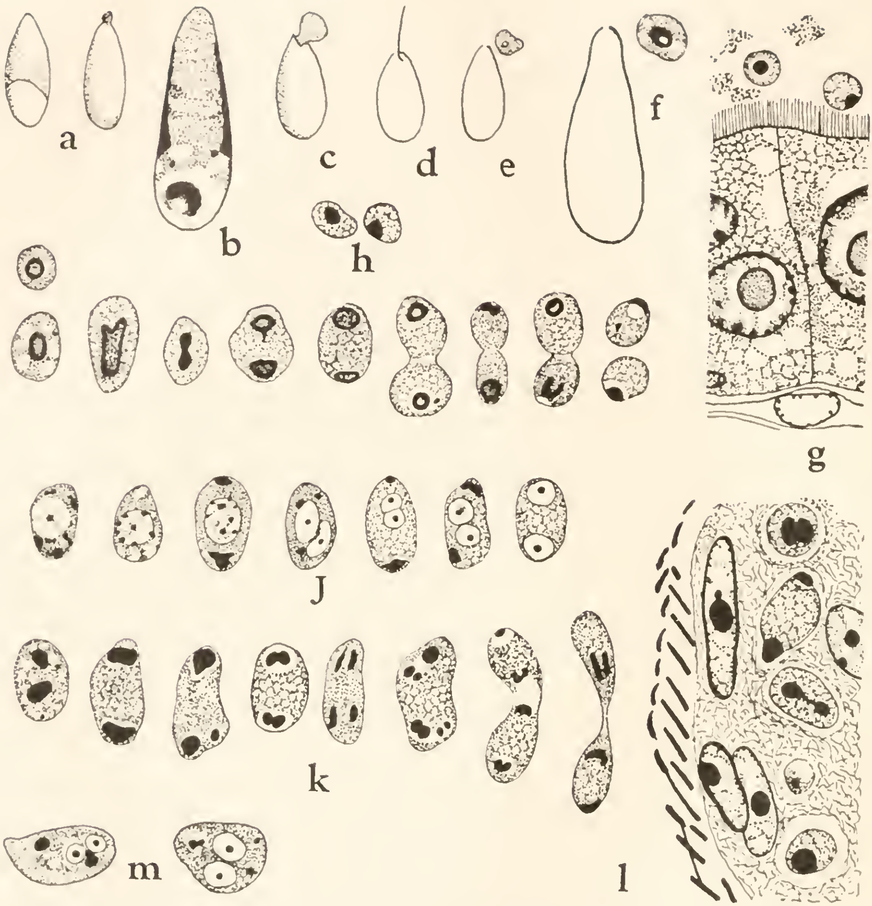

FIG. 1. Developmental stages of *Stempellia magna* observed in the host larvæ fed on infected material. *a, c–e,* × 1000; others, × 2300. *a,* fresh spores taken from the mid-gut of a larva six hours after feeding on the infected material. *b,* a stained spore observed in the same host individual (Giemsa). *c,* a fresh spore observed in the contents of the mid-gut of a larva 24 hours after feeding on the infected material. *d,* a spore found in the mid-gut contents of a larva, 40 hours after feeding on the infected material. *e,* a spore found in the same larva (empty spore membrane and the sporoplasm?). *f,* an empty spore and a uninucleated sporoplasm (?) from the mid-gut of a larva, 24 hours after feeding on the infected material (section; Heidenhain). *g,* two liberated sporoplasms (?) in the lumen of mid-gut of the same larva (Heidenhain). *h,* two young schizonts observed in the periintestinal fat body, 24 hours after feeding. *i, j, k,* schizonts in various stages of development and division as found in the fat-body surrounding the posterior part of the mid-gut and tracheæ in the larvæ examined 24 hours to four days after feeding on the infected material (sections; Heidenhain). *l,* five schizonts in the peritracheal adipose tissue near the mid-gut of a larva, 48 hours after feeding on the infected material (Heidenhain). *m,* the most advanced schizonts (sections; Heidenhain).

immersion objective, but the emergence did not advance any further. I cannot determine whether or not the form changed during this period, since the spore underwent Brown's movements which were further exaggerated by the turning of the finer adjustment of the microscope. Some spores extruded while under observation the filament from the attenuated extremity. At the moment of the extrusion of the filament, the spore showed a vigorous vibration which seemed to have been caused by the sudden unwinding and extrusion of the coiled filament.

In the larvæ which were examined 40 hours after feeding on the infected material, the mid-gut contained more empty spores than those with normal unchanged appearances. Some spores were seen with extruded filaments (Fig. 1, *d*). A number of small amœboid bodies were found in the vicinity of empty spores (Fig. 1, *e*). This amœboid body when first noticed was near three empty spores. It contained a small dark spot near the center. While under observation, I saw it undergoing a sluggish change of form. In the mid-gut of a larva which was fixed 24 hours after feeding on the infected material, uninucleated bodies were seen near empty spores (Fig. 1, *f*).

Since I was not able to completely follow the emergence of the sporoplasm, I cannot state positively that these uninucleated bodies were emerged sporoplasms. The fact that they appeared in a larger number as the empty spores increased and that they resembled closely in size and appearance to the intrasporal sporoplasms and to the youngest stages found in the periintestinal adipose tissue of the host larvæ, however, leads me to consider them as the sporoplasms which left the spores under the influence of the digestive fluid of the host. These amœbulæ are mostly found in the posterior portion of the mid-gut where a number of them were found between the peritrophic membrane and the gut epithelium (Fig. 1, *g*). How these amœbulæ reached the adipose tissue could not be determined, although search was made repeatedly.

The youngest schizonts found in the adipose tissue surrounding the mid-gut and the adjacent tracheæ are shown in Fig. 1, *h*, *i*. They were observed in the larvæ as early as 24 hours after feeding on the infected material. Round in form, it shows reticulated cytoplasm and a large and compact nucleus, the chromatin granules appearing to accumulate in a peripheral layer (Fig. 1, *h*). The nucleus seems to undergo a direct division and forms two daughter nuclei (Fig. 1, *i*). The cytoplasmic body of the schizont grows at the same time, becomes constricted into two parts (Fig. 1, *i*) and finally divides into two uninucleated bodies. This division is most probably repeated in the early phases of the infection, the forms shown in Fig. 1, *l*, being mainly of this kind. The nucleus may sometimes show a karyosome in it; in such a nucleus the division seems to be initiated by that of the karyosome. Frequently the cytoplasm does not follow the nuclear division and the nuclei divide again (Fig. 1, *k*). This is usually followed by an elongation of the body and by a division into two portions in each of which two nuclei are to be found (Fig. 1, *k*).

Another type of division noticed was initiated by a great increase in the size of the nucleus (Fig. 1, *j*). The nucleus becomes vesicular and exhibits a distinct karyosome near its center from which achromatic threads radiate toward the periphery. The cytoplasm contains two or more deeply staining grains. The karyosome divides into two and a septum is formed between them while the deeply staining grains become condensed at the opposite ends. This nuclear division does not seem to be followed by immediate division of the cytoplasm.

As the result of these schizogonic divisions, stages such as shown in Fig. 1, *m*, are produced. These are the only forms which were observed even in the larvæ examined four days after feeding on the material. As the time after feeding elapsed, the number of the various stages of the schizonts present in the fat body increased, although in none of the larvæ stages of sporogony were observed. It may be of interest to note that in one of the larvæ fixed four days after the experiment was started, the follicular epithelium of the ovary on the left side of the host body was greatly replaced by these stages described here, although the young ova seemed to be free from the parasite.

Schizogony.

Young forms found in the adipose tissue of naturally infected larvæ are represented by Fig. 2, *a*. They are comparable with the late stages noted in the experimentally infected larvæ in that both possess two nuclei characterized by a karyosome and deeply staining reticulated cytoplasm. The deeply staining grains in the schizonts (Fig. 1, *m*) already described, may become dispersed in the cytoplasm, though occasionally one sees similar bodies in the naturally infected forms (Fig. 2, *b*).

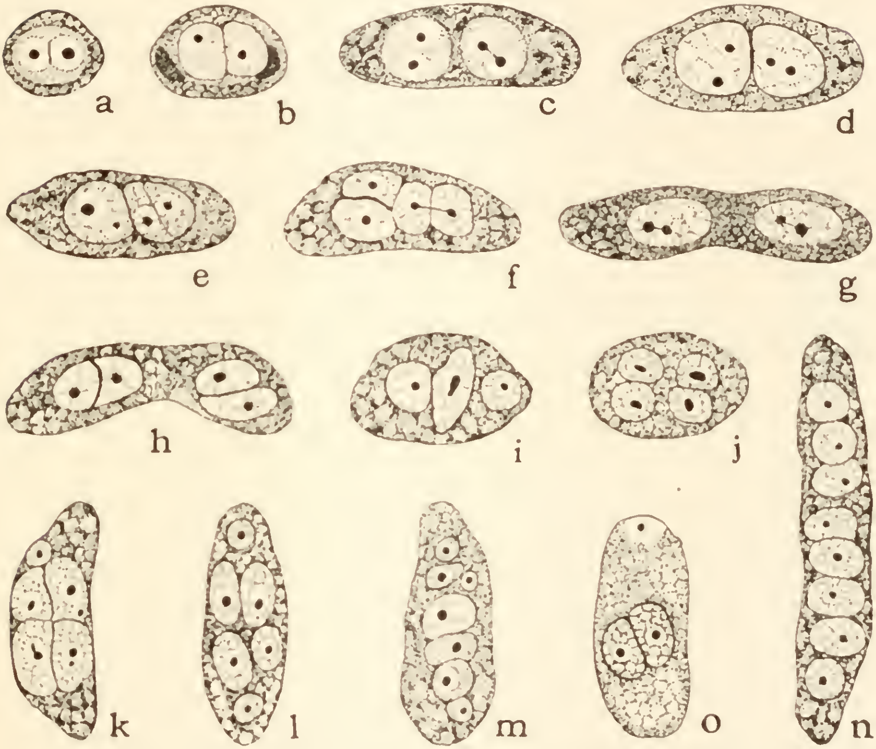

FIG. 2. Schizogonic stages of *Stempellia magna* observed in the sections of the host larvæ naturally infected. Heidenhain × 2300. *a–h*, further developmental stages of schizonts. *i–n*, stages which develop into multinucleate schizonts. *o*, the probable final stage of schizogonic multiplication.

The schizont grows large and the karyosome divides into two (Fig. 2, *c*, *d*) which is followed by the appearance of a membrane between them (Fig. 2, *e*, *f*, *h*). These changes result in forming two binucleated bodies (Fig. 2, *h*) which by further cytoplasmic division form two individuals such as shown in Fig. 2, *o*. The nuclei in the schizonts divide repeatedly without cytoplasmic constriction forming oblong schizonts possessing 3, 4, 5, 6, 7 and 8 nuclei (Fig. 2, *i–n*). These multinucleated forms seem to divide ultimately into binucleated forms (Fig. 2, *o*).

From the 1919 material I described both binary fission and multiple division of the schizonts which produced uninucleated forms; but a study of the preparation stained with Heidenhain's stain and also of the large number of 1920 preparations, leads me to think that the resulting form of the schizogonic divisions is a binucleate form such as are shown in Fig. 2, *o*.

As the two nuclei of the schizont come in a close contact, each karyosome buds off small chromatin granule which seems to be extruded into the cytoplasm later (Fig. 3, *a*). The nuclear membranes between the two nuclei disappear, while the two karyosomes become fused into one. The chromatin grains that were thrown out into the cytoplasm seem to divide further (Fig. 3, *b*).

This uninucleate body is the sporont and gives rise to spores through sporogonic development described below. The fusion of two "cousin" nuclei observed in *Thelohania legeri* (Kudo, '24) does not exist in the present species. Debaisieux ('19) describes a similar change in the schizonts of *Thelohania varians*. Other references on this point are omitted here, since it was brought up in detail in one of my recent papers (Kudo, '24).

Sporogony.

After growing somewhat in size the uninucleate sporont divides. Its karyosome divides into two which become separated by a nuclear wall and the two nuclei are formed (Fig. 3, *c*, *d*). The nuclei become separated from each other and locate themselves near the opposite extremities. A septum appears in the cytoplasm, and two sporoblasts are formed (Fig. 3, *d*). Each sporoblast develops into a spore.

Frequently the two daughter nuclei divide once more. The division begins with that of the karyosome, a strand remaining usually between the divided karyosomes. Thus a tetranucleated sporont is formed; the cytoplasm, as in the case of bisporoblastic sporont, divides into four sporoblasts (Fig. 3, *f*), each of which develops into a spore.

Less frequently a sporont nucleus divides three times, thus producing eight sporoblasts (Fig. 3, *g*) which later develop into eight spores.

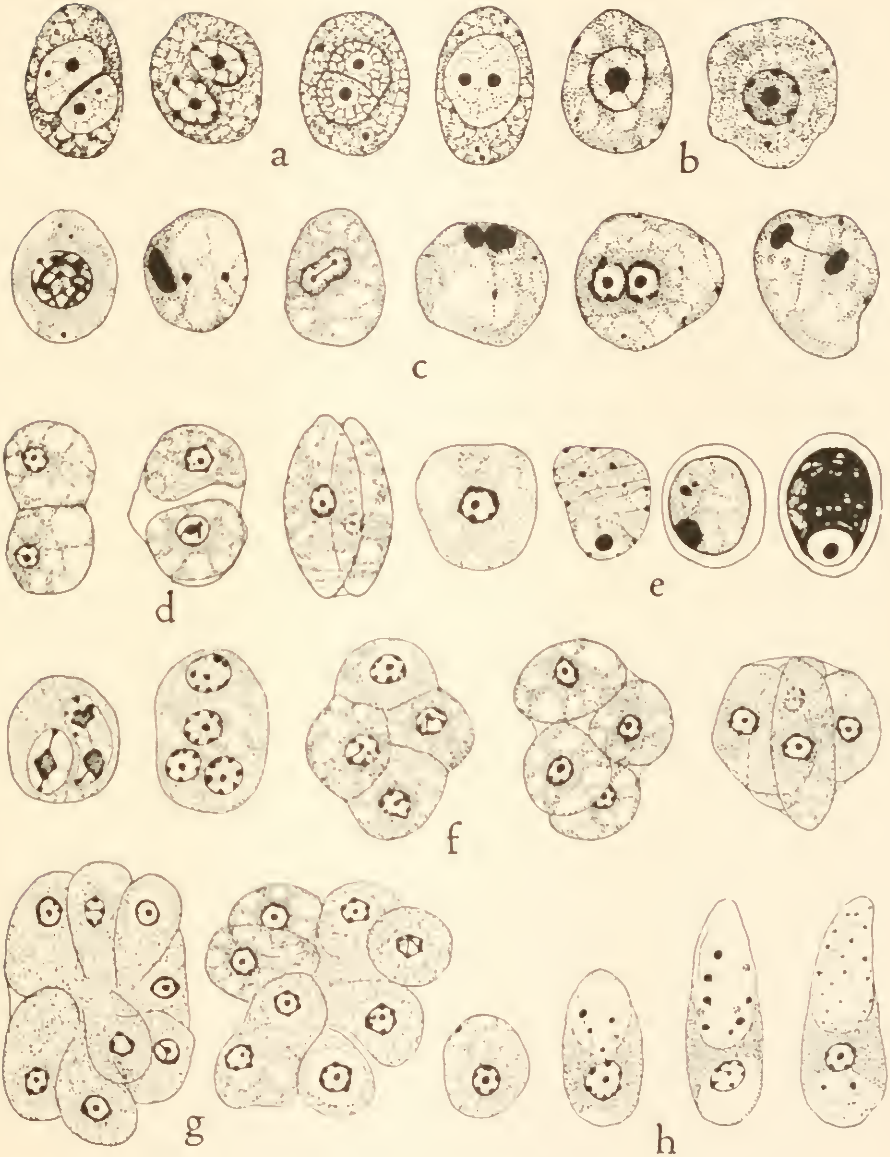

FIG. 3. Further developmental stages of *Stempellia magna* observed in the sections of the host larvæ naturally infected. Heidenhain. × 2300. *a*, four stages in the fusion of the two nuclei of the schizont which results in the formation of sporonts (*b*). *b*, sporonts. *c*, *d*, stages in disporoblastic sporogony. *e*, stages in monosporoblastic sporogony. *f*, stages in tetrasporoblastic sporogony. *g*, stages in octosporoblastic sporogony. *h*, stages in the development of a spore from a sporoblast.

Quite frequently the sporont when discharged into the body cavity of the host transforms into a sporoblast and later into a spore without any nuclear division stated above. This process probably is responsible for the production of abnormally large spores (Fig. 3, *e*).

The cytoplasm of the sporont is more vacuolated and less deeply stained than that of the schizont, of which I remarked before ('21). Schröder ('09) and Schuberg and Rodriguez ('15) mentioned a similar difference in the cytoplasm between the schizonts and sporonts of the Microsporidia they studied.

The sporoblast which varies greatly in size as the natural sequence of the difference in its production, is rounded or oval in form. It has a nucleus composed of peripheral chromatin grains and a karyosome. There is to be seen one or more chromatin grains near one end. The nucleus moves toward the other end of the sporoblast, while deeply staining granules appear in the clear space at the other extremity. These granules become smaller in size and larger in number as the filament is formed, which probably indicates that they are used for the formation of the polar filament. When nearly formed, the spores present the appearance shown in figure 3, *h*; the sporoplasm with one nucleus is near the round end and the coiled filament is present near the other extremity of the spore.

From the sporogony described above, it becomes obvious that the microsporidian cannot be placed in the genus *Thelohania* which is characterized by an octosporous sporont, as I held at first ('20, '21, '22), but should be placed in the genus Stempellia which Léger and Hesse ('10) established for *Stempellia mutabilis*.

THE SPORE

In fresh state, the fully formed spore is elongated pyriform, often bent slightly toward one side. In cross-section it is circular. One end which is ordinarily called as the posterior end, is rounded, while the other, the anterior end, is less rounded, though not attenuated. The spore is moderately refractive and presents somewhat varied aspects. In a large number of spores, there is to be seen an oval, cap-shaped or round area, through which a fine protoplasmic strand sometimes runs transversely (Fig. 4, *c*), while the other part is finely granulated and shows fine irregular lines of coiled polar filament. In some spores, there is no clear space here noted and in others which apparently possess thin spore membrane, numerous transverse protoplasmic strands are to be seen (Fig. 4, *e*).

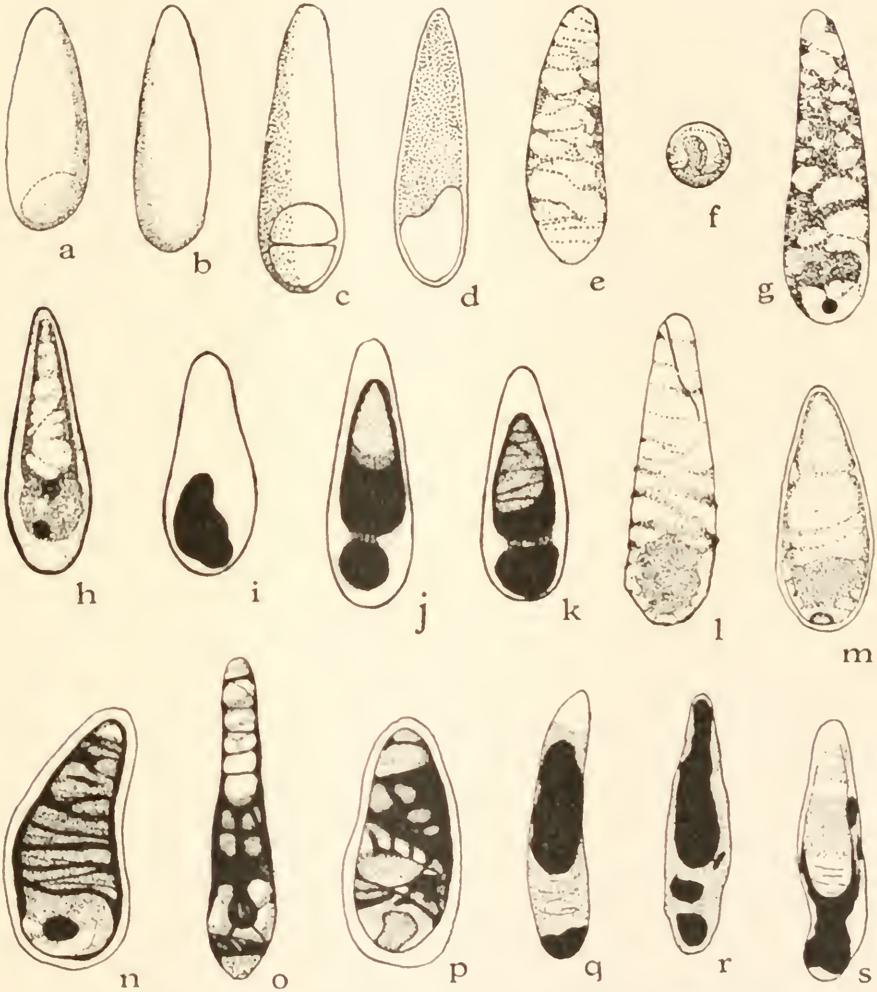

FIG. 4. Spores of *Stempellia magna*. *n–p* from sections; the rest from smears. × 2300. *a–e*, fresh spores. *f*, an end view of a fresh spore. *g*, *h*, spores stained with methylene blue M.P. and observed immediately afterwards. *i–k*, spores kept in methylene blue M.P. for three years under vaselined coverglass. *l*, *m*, spores pressed moderately and kept in Lugol and gum-arabic mixture for two days. *n–p*, abnormal spores, products of monosporous development (Heidenhain). *q*, *r*, normal spores (4 per cent. formol; Giemsa). *s*, normal spore (4 per cent. formol; Heidenhain).

The dimensions of the spore vary to a greater extent than those which I recorded from 1919 material. In fresh state, the spores measure 12.5 to 16.5 μ in length by 4 to 4.6 μ in largest breadth. Some abnormally large spores reach 25 μ by 10 μ, which are without doubt the products of monosporous sporogony.

When the spores are treated with methylene blue, there appears a deeply stained round body surrounded by less deeply staining cytoplasm which I hold as the sporoplasm, while in the remaining part, an irregular network becomes distinctly visible which is the coiled filament. In larger spores, the polar capsule does not seem to be present (Fig. 4, *g*); in the smaller ones, however, it is distinctly recognizable (Fig. 4, *h*, *j*, *k*). When the spores are kept in methylene blue, the polar capsule apparently shrinks and one sees the latter and a rounded sporoplasm in them (Fig. 4, *j*, *k*).

When the spore is subjected to mechanical pressure the filament becomes extruded. The average length of the filament is considerably greater than that obtained from 1919 material for which I gave 150 to 200 μ as the average length. Measurements of a larger number of spores with extruded filaments show that they average 350 to 400 μ in length. Except its base, the filament is of a uniform thickness which is less than one third of a micron in pressure-Fontana-preparations. When the pressed spores are mounted in a Lugol-gum arabic mixture and left for two days, the spore membrane, the sporoplasm and the extruded filaments take yellowish coloration. In such a preparation, the filament is considerably thicker, as a result of the swelling due to the medium used for the mounting (Fig. 5, *a*).

Contrary to some authors, there is no thickening at the distal end of the filament of the present form as was the case in all the other species of Microsporidia which I have studied up to the present (Fig. 5, *f*). Very rarely one sees a thick point at the extremity of the extruded filament (Fig. 5, *c*), an examination under a higher magnification shows, however, that here the filament became probably broken during the extrusion and the material which compose the filament became spread out as a result (Fig. 5, *e*).

Abnormal spores such as shown in Fig. 4, *n–s* are of frequent occurrence. Normal spores appear irregularly slender in form when fixed in smears with formol (Fig. 4, *q–s*).

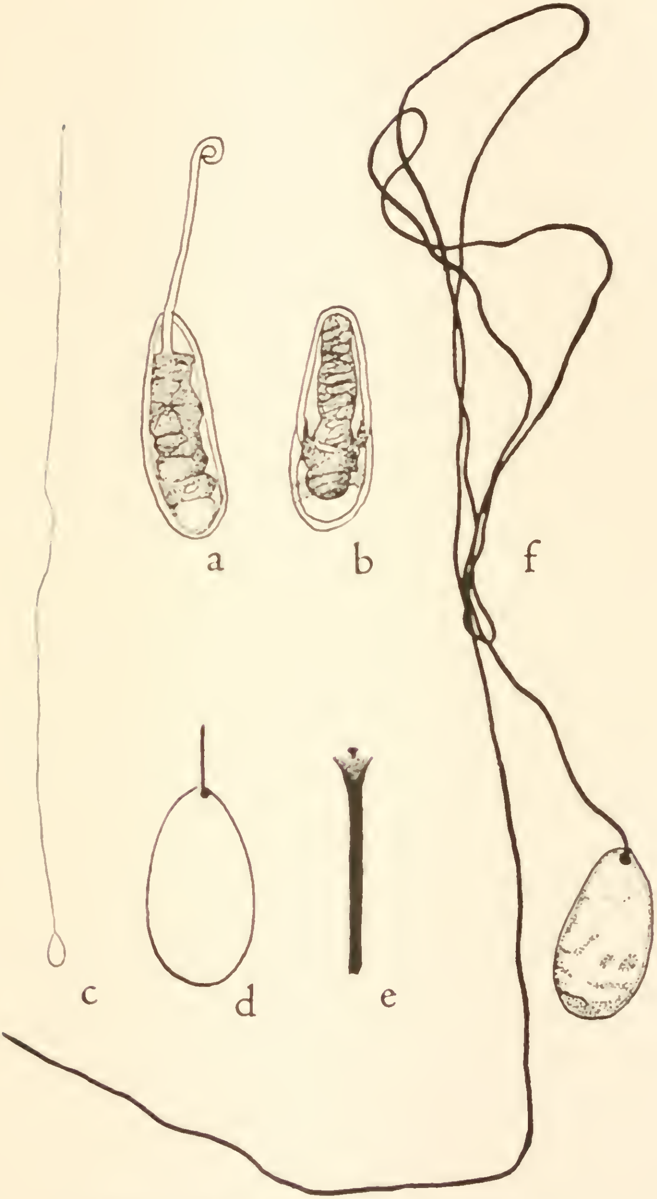

FIG. 5. Spores and polar filaments of *Stempellia magna*. Smears. *c*, × 360; *e*. × 3200; the rest, × 2300. *a*, *b*, spores pressed mechanically and kept in Lugol and gum-arabic mixture. *c*, a spore pressed mechanically and stained after Fontana. *d*, the same spore under higher magnification. *e*, the distal end of the filament shown in *c* highly magnified. *f*, a spore similarly treated, but stained with Giemsa.

THE RELATION BETWEEN THE PARASITE AND THE HOST.

I have remarked about this subject before (Kudo, '21, '22) and do not at present have any additional statement to add. So far I have failed to observe adult mosquitoes infected by the protozoön. But from what I have recently seen in the cases of infection of adult anopheline mosquitoes by *Thelohania legeri* and *Nosema anophelis* in Georgia, it is quite possible that the Culex larvæ lightly or moderately infected by *Stempellia magna* would be able to metamorphose into adults; on the other hand, when the infection of the host larvæ by the microsporidian is to such an extent as to show a typical symptom to unaided eyes, the host larva would die before completion of larval life.

SUMMARY.

1. *Stempellia* (*Thelohania*) *magna* was found to be parasitic in the larvæ of *Culex pipiens* (Illinois, 1919) and of *C. territans* (Pennsylvania, 1920).

2. The infection experiment shows that the larvæ become infected by feeding upon the infected larval tissue.

3. The emergence of the sporoplasm of the spore taken into the gut lumen of a new host takes place in the posterior part of the mid-gut from 6 to 40 hours after feeding on the infected material.

4. The schizonts are first noticed in the adipose tissue of the mid-gut and of adjacent tracheæ.

5. The sporogony did not start in the larvæ examined four days after feeding on the infected material.

6. Schizogony is a binary fission of various types. The final form is binucleated. The two nuclei undergo autogamy, forming a sporont.

7. The sporont develops into ordinarily two, frequently one or four and rarely eight sporoblasts; these develop into two, one, four and eight spores.

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A MUTATION IN THE MOTH-LIKE FLY (*PSYCHODA ALTERNATA*) AND THE METHOD OF ITS TRANSMISSION.

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I. Life History and General Features of the Normal Fly.

The Moth-like Flies are inconspicuous insects about 2 mm. in length which breed in water, decaying vegetation and in manures. They have been little studied and consequently the life histories of only a few are completely known. In one or two instances they have been observed under laboratory conditions for two or three generations with the end in view of determining the lengths of the various stages of the life histories. There is no record, however, of their being carried under such conditions for more than a few generations.

During the past year the writer has reared successfully two species (*Psychoda alternata* and *Psychoda minuta*) and one of the two has proved itself to be an excellent subject for studies in heredity. A mutation has arisen in *Psychoda alternata* and the method of its transmission determined. A full account[1] of the life histories and of the general features of the two species mentioned has been offered in a previous paper so that no extended account will be necessary here. It seems necessary, however, to repeat the account in so far as it has to do with the mutation.

Eggs are laid upon moist manure by the females and within a few days they hatch producing active, feeding larvæ resembling those of midges. The larvæ possess pigment in two forms. Chitinous plates are present on the dorsal side of the last two or three segments (Fig. 1, *C.P.*) and a heavy chitinous sheath covers the head and the last segment (Figs. 1 and 2). These plates are light brown in the young larvæ but they grow darker with age. The other pigment is reddish brown in color and is found in the ocelli (Fig. 2, *Oc.*) and the Malpighian tubules of the larvæ (Fig. 1., *M.P.T.*).

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[1] The Psychodidæ as Subjects for Studies in Breeding and Genetics, *Amer. Nat.*, Vol. LVII, pp. 545–558.

After eight days the larva becomes quiescent and pupates, spending two days in the pupal stage. The chitinous coverings become more extensive during this time and much darker, especially over the head and the thorax (Fig. 3). The Malpighian tubules become shorter and the reddish brown pigment in them becomes somewhat darker (Fig. 3, *M.P.T.*). During the pupal stage the compound eyes, each containing about seventy-five facets, develope. The same reddish brown pigment that occurs in the ocelli and in the Malpighian tubules also developes in the facets of the compound eyes. The chitinous walls between the facets become darkly colored but only after the reddish brown pigment of the facets is thoroughly established. During the formation of the compound eyes the ocelli degenerate.

The adult emerges as an extremely hairy insect with pigment present in three forms. The compound eyes (*CE.*, Figs. 4 and 5) and the Malpighian tubules (Fig. 4, *M.P.T.*) contain the reddish brown pigment first observed in the larval ocelli and Malpighian tubules. The chitinous covering of the body is colored with browns of various degrees of intensity and the hairs which cover the body and the wings range in color from white to brown and black. Especially on the wings the color changes of the hairs are so abrupt as to give a mottled appearance. Small patches of hairs, coarse and black, and very conspicuous are located at the ends of the wing veins.

The adult life lasts from two to nine days, depending upon the sex and upon the event of copulation. All normal adults are positively phototropic and negatively geotropic when they first emerge but become noticeably less sensitive to light and to gravity as they grow older. These two reactions play an important rôle in bringing the two sexes together for copulation. The adults are apparently sexually mature at the time of emerging and copulation takes place within a few hours. In copulation the male seizes the female with a pair of heavy terminal forceps.

II. Character of the Mutation.

The mutation varies from the normal fly in three phases of its structure and in its reaction to light. All these differences may be traced to a single scource, namely, the lack of the reddish brown pigment in all of those structures in which it ordinarily occurs in the normal fly. This lack is first evident in the larva where the ocelli are colorless (Fig. 7, *Oc.*) and the cells of the Malpighian tubules are also devoid of color (Fig. 6). In the Malpighian tubules of the mutant fly there are granular bodies which in the normal fly bear the pigment but are here colorless. In the pupal stage the Malpighian tubules go through the same structural changes as in the normal fly but no pigment develops in the granular bodies. Neither do the ocelli of the mutant which up to this time have developed no pigment become colored. The compound eyes of the mutant which are formed at this stage are normal except that they, too, develope no pigment (Figs. 9 and 10). In the adult fly the reddish brown pigment is wholly lacking in the compound eyes (Fig. 10, *C.E.*) and in the Malpighian tubules (Fig. 10).

The other phases of coloration are not affected. The chitinous coloration in the larva, pupa and adult stages cannot be distinguished from that of the normal specimens. The pigment coloration in the chitinous walls of the compound eyes is entirely obscured in the normal fly by the heavier coloration in the facets but in the mutation, because of the lack of the reddish brown pigment in the facets, the coloration of the chitinous interspaces is very prominent (Figs. 9 and 10). The coloration of the hairs of the mutant is identical with that of the normal fly.

In their reactions to gravity the normal and mutant flies behave alike but there is a marked difference in their phototropic reactions. The normal brown-eyed specimens have a strong positive reaction to light while the white-eyed ones are practically indifferent to light of the same intensity. Strong light has a kinetic effect upon both normal and mutant flies.

III. Nature of the Determiner.

If a single determiner be postulated for the lack of the reddish brown pigment which appears in turn in the simple eyes of the larva, the Malpighian tubules of the larva, pupa and adult and the compound eyes of the pupa and adult it would seem to be sufficient to account for all phases of the mutation. The fact that the pigment occurs in all three phases or not at all would argue for such a determiner. If any case had arisen in which the reddish brown pigment occurred in one phase and not in another it would necessitate the postulating of a determiner for pigment in each phase. This series of pigmentary phases greatly facilitates the work of identifying progeny since specimens may be positively identified as normal or mutation in larval, pupal or adult stages.

In some cases of inheritance the determiner for a character does not become expressive except at a single stage in the development of the animal. Usually it is in the adult stage that a determiner shows its influence. The determiner must necessarily be present in all stages up to the time of its expression but may be regarded as only a potential influence until the proper time is reached. It is interesting to note that in the present case the determiner exerts its influence at three separated stages in the animal's life history; in the formation of the ocelli, in the development of the Malpighian tubules and finally in the formation of the compound eyes. This would indicate that some general metabolic process was being interfered with whenever that process was operating. In this particular case, then, the determiner might be described as a regularly inherited "influence" upon the metabolic processes which are necessary to the elaboration of the reddish brown pigment.

IV. METHOD OF TRANSMISSION OF NON-PIGMENTED CONDITION.

When it was first discovered that white-eyed mutants were appearing in a mass culture the culture was closely watched and the mutants were isolated as they appeared. From the number of specimens appearing it became evident that the generation in which they had been discovered was not the one in which they had arisen. It is quite probable that one of the specimens first seen and confined in the jar was a white-eyed individual which had copulated with one or more of the normal specimens confined with it.

The mass culture in which the mutants arose would in such a case be a mixture and without knowing the method of inheritance of the mutation it would be impossible to know whether either brown- or white-eyed specimens were homozygous for the character in question. Neither was it known whether the variation would be permanent and heritable or whether it would prove to be a fluctuating variation.

A. PRELIMINARY EXPERIMENTS.

Using the white-eyed and brown-eyed specimens with unknown genetic constitution from the mass culture preliminary crosses were made for the purpose of getting some information as to the heritability and possibly the method of inheritance of the mutation in case it proved to be heritable.

a. White-eyed by White-eyed Crosses.

Sixteen crosses of white-eyed by white-eyed specimens were made, the specimens being isolated at the time of their emergence to insure virgin stock. From all these crosses 731 individuals were obtained and all were white-eyed. The eyes and Malpighian tubules of all showed a total lack of pigmentation. The question as to the heritability of the mutation was thus answered while the completeness and the permanence of the mutation has subsequently been established by maintaining pure strains of white-eyed flies up to the present time.

b. Brown-eyed by Brown-eyed Crosses.

Brown-eyed flies were isolated from the mass culture in a very young stage and 163 crosses were made, 91 of which were successful. In some cases all of the resulting progeny were brown-eyed. For example, culture number 138 produced three brown-eyed males on the ninth day, fifty-three more on the tenth day and during the following four days twenty-two brown-eyed males and forty-seven brown-eyed females.

In a few cultures some white-eyed specimens appeared. All such cultures showed a comparatively large number of brown-eyed specimens but the ratio was variable. It was important, however, to have obtained white-eyed specimens from a brown-eyed by brown-eyed cross since it indicated that the brown-eyed condition was probably dominant and the white-eyed condition recessive.

c. Brown-eyed by White-eyed Crosses.

Twenty-eight matings were made using brown-eyed males and white-eyed females and twenty-four using brown-eyed females and white-eyed males. Both types of crosses produced the same result thus demonstrating that there was no sex linkage. Of these matings thirty-two were successful. In six lots of progeny all specimens were brown-eyed. The remaining lots contained both brown-eyed and white-eyed progeny, the white-eyed varying in proportion from 27 to 58 per cent. of the total number in each lot.

d. Discussion of Results in Preliminary Crosses.

The results of the preliminary crosses indicated that the normal condition was behaving as a dominant and the mutation as a pure recessive. Assuming that the normal condition (*BB*) was dominant and the mutant condition (*bb*) was recessive six possible crosses could be made and the expectations in the progeny would be as follows:

Crosses.	Expected Ratios (Phenotypes).
bb × *bb*. .	100 % white-eyed
BB × *BB*	100 % brown-eyed
BB × *Bb*	100 % brown-eyed
Bb × *Bb*	75 % brown-eyed, 25 % white-eyed
BB × *bb*	100 % brown-eyed
Bb × *bb*.	50 % brown-eyed, 50 % white-eyed

The full expectation was realized in the preliminary *bb* by *bb* crosses.

Since both pure brown-eyed (*BB*) and hybrids (*Bb*) were contained in the mass cultures from which the breeding stock was selected it would be expected that the brown-eyed by brown-eyed crosses might be either *BB* by *BB* or *BB* by *Bb* in which case all of the first filial generation would be brown-eyed; or they would be *Bb* by *Bb* in which case there would be expected a large proportion of brown-eyed individuals and a few white-eyed ones in the first filial generation. This expectation was also approximately realized in the preliminary brown-eyed by brown-eyed crosses in that a small proportion of white-eyed specimens were procured from such crosses.

In the brown-eyed by white-eyed crosses there were two possibilities, namely, that the brown-eyed specimens used were either pure (*BB*) or hybrids (*Bb*). If the brown-eyed parents were pure all the progeny of the first filial generation would be brown-eyed. If the brown-eyed parents were hybrids then 50 per cent. of the first filial generation would be white-eyed. This expectation was realized in some lots as far as the securing of all brown-eyed progeny from brown-eyed by white-eyed crosses. However, the expectation of 50 per cent. white-eyed and 50 per cent. brown-eyed progeny was only approximately realized in the preliminary experiments.

B. EXPERIMENTS WITH PEDIGREED STOCK.

The strains used in these experiments were selected from vigorous stock all the members of which had been recorded through several generations. The white-eyed strain was one which had been started soon after the discovery of the white-eyed mutation and one which had been inbred from the start. The brown-eyed stock was one which had produced only brown-eyed individuals for eight generations.

a. White-eyed by White-eyed Crosses.

All progeny secured from white-eyed pedigreed stock was white-eyed. About twenty-two thousand of these individuals have been recorded.

b. Brown-eyed by Brown-eyed Crosses.

Pure brown-eyed by pure brown-eyed crosses have produced only brown-eyed progeny. About sixteen thousand of such individuals have been recorded.

c. Brown-eyed by White-eyed Crosses.

Whenever pure brown-eyed were crossed with pure white-eyed individuals the resulting progeny were invariably brown-eyed. About twelve thousand such progeny have been recorded.

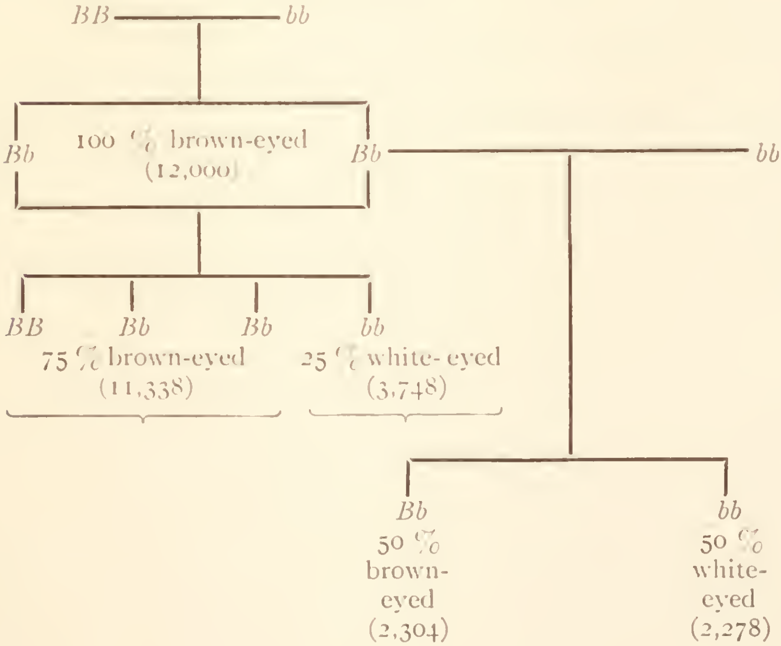
In the second filial generation of pure brown-eyed by pure white-eyed crosses 15,086 individuals were obtained from 167 successful crosses. Of this total number 11,338 were brown-eyed and 3,748 were white eyed.

Back crossing the brown-eyed hybrid of the first filial generation with the pure white-eyed parental stock was carried out successfully thirty-seven times giving a total of 4,582 progeny of which 2,304 were brown-eyed and 2,278 were white eyed.

d. Discussion of Results of Experiments with Pedigreed Stock.

The results obtained verify the supposition that the condition represented by pigmented compound eyes, Malpighian tubules and ocelli is dominant and the condition representing a lack of pigment is recessive. Since the results were uniformly independent of sex it follows that there is no sex linkage. The ratio expected in the second filial generation of a pure brown-eyed by pure white-eyed cross would be three brown-eyed to one white-eyed individual. The ratio realized (3.032 to 1) is near enough to the theoretical ratio to demonstrate that Mendels Law is operative. This is still further supported by the results from back crossing heterozygous brown-eyed stock with homozygous white-eyed stock. In terms of Mendel's Law it would be expected that one half of the progeny would be white-eyed and one half brown-eyed. A close approximation to this ratio was realized when out of a total of 4,582 individuals 2,304 were brown-eyed and 2,278 were white-eyed.

Following is a diagram illustrating the correlation of experimental results with expectations according to Mendel's Law for brown-eyed by white-eyed crosses. The number of individuals obtained in the experiments are shown in parentheses.



EXPLANATION OF FIGURES.

Note: All photographs were made from specimens killed and cleared in absolute alcohol and xylol and mounted in balsam. No stains were applied and none of the photographs have been retouched.

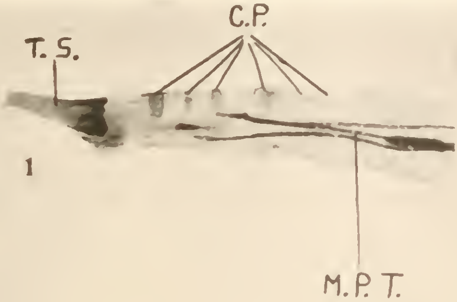
FIG. 1. Photomicrograph of the last four segments of a normal 10 mm. larva illustrating the Malpighian tubules (*M.P.T.*), the heavily chitinized terminal segment (*T.S.*) and the light dorsal chitinous plates (*C.P.*). × 24.

FIG. 2. Photomicrograph of the head of a normal larva illustrating the pigmented ocellus (*Oc.*). × 140.

FIG. 3. Photomicrograph of a normal pupa illustrating the Malpighian tubules (*M.P.T.*). The pigmented compound eyes are obscured by the dense chitin of the head and thorax. × 80.

FIG. 4. Photomicrograph of a normal adult male fly. In bringing the Malpighian tubules (*M.P.T.*) into sharp focus the compound eyes (*C.E.*) were somewhat blurred. × 30.

FIG. 5. Photomicrograph of the face of a normal adult fly illustrating the pigmented compound eyes (*C.E.*). The separate facets cannot be made out because of the uniform dense pigmentation. × 120.



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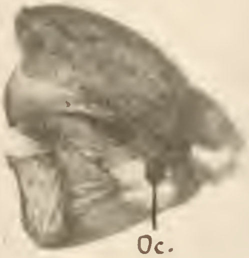

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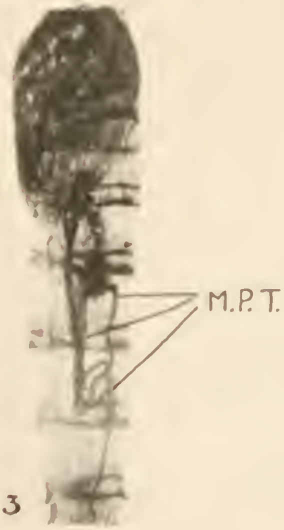

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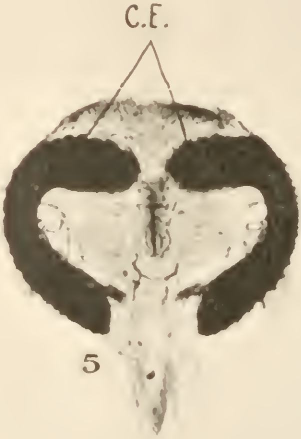

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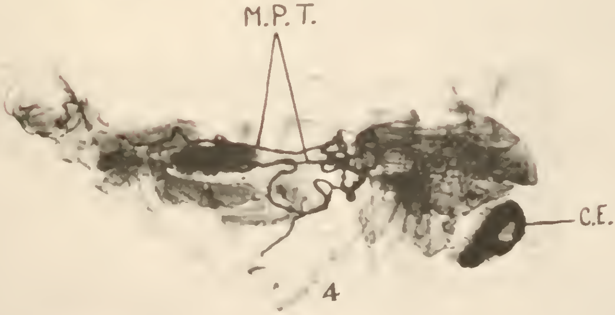

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FIG. 6. Photomicrograph of the last four segments of a mutant larva. The Malpighian tubules are present but as they bear no pigment they do not appear in the photograph. × 21.

FIG. 7. Photomicrograph of the head of a mutant larva. The ocellus (*Oc.*) is not pigmented and so does not show in the photograph. × 140.

FIG. 8. Photomicrograph of the face of a mutant pupa illustrating the compound eyes (*C.E.*) which contain no pigment. × 100.

FIG. 9. Photomicrograph of the face of a mutant adult showing the compound eyes (*C.E.*). The margins and the interspaces have a yellowish-brown color that is characteristic of the heavily chitinized parts of the body but there is none of the dark reddish brown pigment in the facets that is found in the facets of the normal fly. × 132.

FIG. 10. Photomicrograph of an adult mutant fly showing the absence of pigment in the compound eyes (*C.E.*) and in the Malpighian tubules. × 30.

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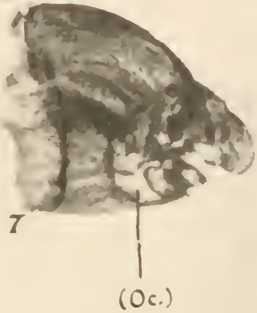

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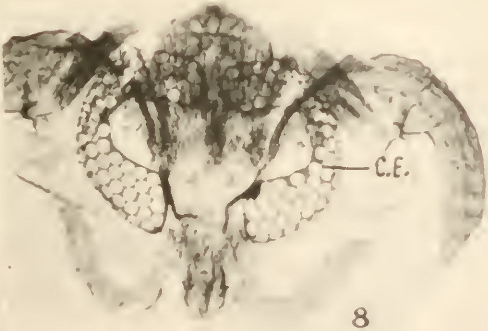

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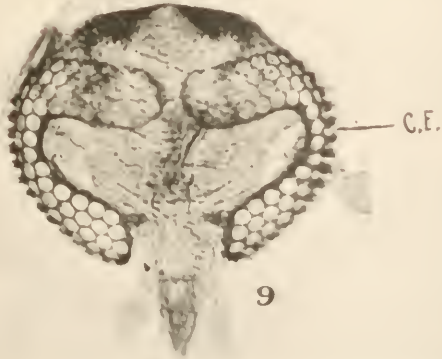

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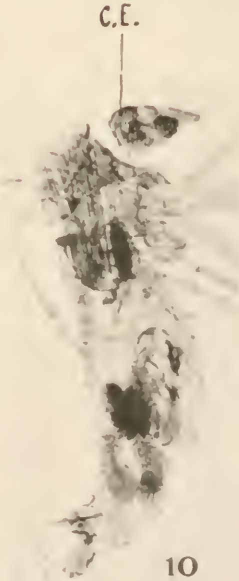

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C. L. TURNER.

REVERSAL OF FUNCTION IN A SPECIES OF *OLIGARCES*.

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There occur in *Oligarces* sp. two methods of reproduction: pædogenesis and adult sexual reproduction. These two methods are accompanied by a corresponding dimorphism among the larvæ of the species. Thus there are (1) pædogenetic larvæ and (2) pupa-larvæ. These two forms are functionally and morphologically different. The former larvæ, as the name implies, reproduce by pædogenesis, while the latter metamorphose into pupæ, which in turn become adults. Among the morphological differences existing between these two larval forms, three are of great value in distinguishing pupa-larvæ from pædogenetic larvæ, and vice-versa, both in the laboratory and in nature. These are (Harris '23):

"1. In newly born living pupa-larvæ the imaginal discs are visible. These occur laterally in the third, fourth and fifth segments. They are not present in pædogenetic forms.

"2. The spathula sternalis, a structure typical of Cecidomyid larvæ, occurs ventrally in the third segment of the pupa-larvæ of *Miastor*. It is not visible in newly born living pupa-larvæ, but after four or five days becomes clearly visible, due to a yellowish coloration which later changes to orange and dark brown. The shape of this structure differs with various species. Its function is not understood.

"3. The eyes of pupa-larvæ do not touch as in pædogenetic forms but are usually clearly separated."

These morphological differences correspond to those which Springer ('17) noted in *Miastor metraloas*. They have been confirmed in that species by the writer, and obtain in all species of *Oligarces* and *Miastor* which I have observed. Though many hundreds of pupa-larvæ, and thousands of pædogenetic larvæ have been examined, none have been noted, either in nature or in the laboratory, in which the morphological appearance of the two larval forms did not correlate perfectly with the function of the larva, under normal conditions. Thus the writer and other observers have used the terms pupa-larvæ, and pædogenetic larvæ as definite terms (Springer '17, Harris '23, '24), based upon visible morphological differences, corresponding absolutely to functional differences. Pædogenetic larvæ consistently give rise, by pædogenesis, to other larvæ; whereas pupa-larvæ never give rise directly to other larvæ, under normal conditions, but continue their development by metamorphosis.

Recently I have indicated, as the result of a series of experiments, that pupa-larvæ may be obtained at will in the laboratory by crowding in the previous generation (Harris, '23, '24). During these studies, counts were made of pupa-larvæ which had arisen from crowded cultures of pædogenetic larvæ. It was found necessary, in order to make accurate counts to remove the pupa-larvæ from the original culture to a fresh culture, at the time of counting. This procedure removed the possibility of counting the same larva twice, and, at the same time, permitted counts to be made as pupa-larvæ arose in the culture; thereby making the larvæ available for other experiments.

Upon examining the abdominal contents of pupa-larvæ which had been transferred to fresh cultures, I observed in one pupa-larvæ not only eggs but young embryos. Both the eggs and young embryos were clearly distinguishable in the method employed.

Pupa-larvæ were placed in Ringer's solution on a slide and the anterior portion of the pupa-larvæ was removed with dissecting needles. The contents of the abdominal cavity were then forced out through the opening thus made; the larval skin was removed and the material was stained with a modified aceto-carmine preparation.[1]

The pupa-larvæ, already mentioned, appeared originally in culture M 187, which had previously been subjected to intense crowding. It, with other pupa-larvæ, was transferred to a fresh culture, MP 142, on December 30, 1923. When examined, as previously stated, 7 days later, the larva was found to contain 4 young embryos and 10 eggs. In the same culture another pupa-larva was observed with two large embryos, while other pupa-larvæ were observed containing varying numbers of embryos, though in no case more than four. On January 8, young larvæ escaped from several mothers, which had all the morphological characteristics of pupa-larvæ. These young, 33 in number, were typical pædogenetic larvæ, and later produced young pædogenetically in their turn. Of the total of 40 pupa-larvæ (which were transferred from culture MP 142), 27 metamorphosed into pupæ, and these in turn into adult flies, while 13, showing a reversal of function, did not metamorphose but gave rise to young in a typically pædogenetic fashion.

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[1] Acknowledgment is made for this staining method to Dr. Belling, of the Carnegie Institution of Washington, who has employed it with success in cytological work on *Datura*.

Since I had already observed that pupa-larvæ could be produced at will in the laboratory from a pædogenetic strain by crowding, it seemed probable that the apparent reversal of function was called forth by a reversal of the external conditions surrounding the pupa-larvæ. Thus the transference of a pupa-larvæ to a fresh culture immediately removed them from the influence of crowding, thus reversing the external conditions to which they had been subjected.

The fact that some of the pupa-larvæ seemed to respond to the treatment, while others did not, suggested the possibility that the age of the larvæ, when transferred, and by consequence the stage of development of the gonads and eggs, determined whether or not a reversal of function would occur.

(It may be said that the material upon which these observations were made is a female producing strain (Harris, 1924). The question then probably resolves itself into whether or not the eggs have reached a stage at which parthenogenetic reproduction is no longer possible.)

In order to ascertain if the apparent reversal of function were due (1) to the release from crowding, and from the consequent presence of an unusually large amount of the by-products of metabolism; or if it were dependent upon (2) the age of the pupa-larvæ when released from crowding, another series of experiments was carried on, using the same stock in the second subsequent generation. In this generation pupa-larvæ arose, as a result of crowding during embryonic developement, in culture M 222, M 223, and M 224. These pupa-larvæ were transferred to fresh cultures, made of the same stock culture medium, and maintained under conditions as similar as possible to those of the control cultures. On this occasion, however, pupa-larvæ were separated into two age-groups.

It will be remembered that the spathula sternalis, a structure characteristic of pupa-larvæ in this material, is unpigmented when the larvæ are born, but becomes pigmented later, until, about three or four days after the pupa-larvæ is born, the spathula sternalis is dark brown in color. Thus the presence or absence of pigment in the spathula sternalis offers a rough index of age. This index was employed. Pupa-larvæ in which the spathula sternalis was unpigmented were placed in one series of fresh cultures, those in which pigmentation was clear were placed in another series. Four such cultures were formed, MPW 190, MPW 192, MPWO 191 and MPWO 193. The even numbered cultures contained only pupa-larvæ in which the spathula sternalis was pigmented; while in the odd numbered cultures none of the spathulæ sternalis was pigmented at the time of transfer. All larvæ were, from a morphological standpoint, typical. 16 pupa-larvæ with spathula sternalis pigmented were transferred to culture MPW 190; and 22 to culture MPW 192. Of the former, 7 metamorphosed into pupæ, and later into adults, while 9 produced young pædogenetically. In culture MPW 192, of the 22 pupa-larvæ present, 13 metamorphosed into pupæ and later into adults, while 9 gave rise to young pædogenetically. It is significant that in the cultures made from younger pupa-larvæ, as evidenced by the absence of pigmentation in the spathula sternalis when the larvæ were transferred, of the 15 pupa-larvæ in culture MPWO 191 only 1 metamorphosed into a pupa, and later into an adult, while 14 produced young pædogenetically. In culture MPWO 193, of the 8 pupa-larvæ present, none continued their development in the manner characteristic of pupa-larvæ, but all showed a reversal of function, giving rise to young in the manner of typical pædogenetic larvæ, though in most cases the number of young born was less than that of pædogenetic mothers from the same stock. All the young which were produced by the pupa-larvæ-mothers were, morphologically and functionally, typical pædogenetic larvæ.

TABLE I.

REVERSAL OF FUNCTION OF PUPA-LARVÆ FOLLOWING THE RELEASE OF YOUNG PUPA-LARVÆ FROM CROWDING.

Origin.	Transferred to Culture.	No. Showing Normal Function.	No. Showing Reversal of Function.	Total no. Pupa-larvæ.	Remarks.
M 187.........	MP 142	27	13	40	Age not considered.
M 222, 3 and 4 .	MPW 190	7	9	16	Spathula sternalis pigmented.
M 222, 3 and 4	MPW 192	13	9	22	Spathula sternalis pigmented.
M 222, 3 and 4..	MPWO 191	1	14	15	Spathula sternalis unpigmented.
M 222, 3 and 4	MPWO 193	0	8	8	Spathula sternalis unpigmented.

Culture M 187 was made up of larvæ of the F 10 generation of a strain maintained in the laboratory, cultures M 222, M 223, and M 224 represent the F 12 generation. This strain was subjected to crowding with the consequent production of pupa-larvæ in five of the ten generations. In no cases in which pupa-larvæ were not released from crowding did reversal of function occur.

In view of the results of this study the conclusion seems to be well founded that a reversal of the normal function of pupa-larvæ to that of pædogenetic larvæ can be obtained at will in the laboratory by reversing the conditions under which pupa-larvæ arose. This reversal has been obtained in the present instance by releasing pupa-larvæ from crowding.

Newly born and very young pupa-larvæ are more likely to give evidence of the influence of changed external conditions than are older pupa-larvæ. This probably indicates that a change, possibly reduction division, occurs in the eggs of the pupa-larvæ when the larva is still young. Before this change has occurred the eggs are still capable of pædogenetic development if external conditions are suitable. The progressive development of embryos within such a pupa-larva probably inhibits the normal function of the larva to an extent that, instead of realizing its normal function, the larva, though originally a pupa-larva, functions as a pædogenetic larva. If the change has been accomplished the eggs will not develop pædogenetically, and the pupa-larva continues its existence in accomplishing its normal life-history.

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Acknowledgment is made to Dr. C. B. Davenport, of the Dept. of Genetics of the Carnegie Institution of Washington, for facilities offered at the station in connection with this work.

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BIOLOGICAL BULLETIN

A MODIFICATION OF THE URODELE TESTIS RESULTING FROM GERM-CELL DEGENERATION.

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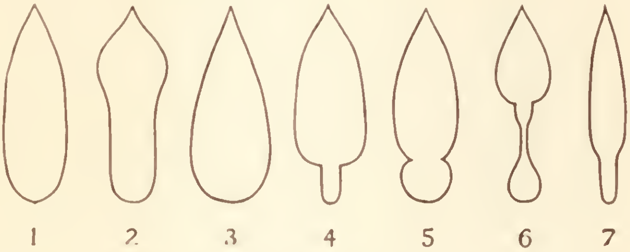
Descriptions of the Urodele testis, particularly those of the earlier investigators (von Wittich, '53; Hoffmann, '73, '78; Spengel, '76) frequently contain references to peculiarities of structure, such as lobation, constriction, or reduction in size of caudal or cephalic ends. The significance of such peculiarities was apparently but little understood or appreciated. Although Spengel makes the brief (and correct) statement that these are but the result of the progressive and regressive changes in the testis, no elaboration of this view serves to show whether the statement was more than a speculation.

The simplicity of the structural plan of the testis in many Urodeles (the *Plethodons*, *Eurycea*, *Desmognathus*, *Gyrinophilus*) makes the organ particularly susceptible to modifications in form. Of an elongated, cylindrical shape (Fig. 1) it is traversed by a longitudinal collecting duct, more or less central in position, around which the chambers or lobules are arranged in either radial or fan-like fashion, as shown in Fig. 8. The synchronous development of all the cells of the lobule, and the caudo-cephalic order of development and extrusion of the germ cells, in a testis of this type, results in normal seasonal variations that would not otherwise be possible. The earlier emptying of its lobules, for example, reduces the size of the caudal portion of the testis as compared with that territory yet containing spermatozoa (Fig. 2). Later, after the final emptying of the anterior lobules, the inequality in size of the two regions may be quite reversed (Fig. 3) due to the earlier growth, in the caudal lobules, of the spermatocytes for the next cycle. It can be readily appreciated, too, that an unusual degeneration of germ cells, limited to any one region, would reduce the size of the testis at that point, producing either a slender extension of the organ (Fig. 4) or a constriction between two regions of greater diameter (Fig. 5). In *Desmognathus* and other Urodeles (*Diemyctylus*, *Salamandra*) the peculiar pattern of spermatogenesis ultimately converts the simple type of testis above described into a "multiple testis," that is to say, a structure consisting of from two to five simple testes joined in series by slender intermediate regions containing only the central duct and a few surrounding primary spermatogonia. Figure 6 indicates in outline the form of a "multiple testis" of two lobes. The origin of this peculiar type of testis has been discussed at length in a separate paper (Humphrey, '22). In other species (the *Plethodons*, *Gyrinophilus*, *Eurycea*) a "multiple testis" has never been encountered by the writer in an examination of hundreds of specimens. The testis remains a simple or unit structure, tapering anteriorly, more or less uniform in diameter throughout its greater extent, and ending caudally either in a bluntly rounded fashion (Fig. 1) or as a slender prolongation, variable in length, which I shall designate as the caudal appendage (Figs. 4 and 8). This structure constitutes the subject of the present study.

Such an appendage will be encountered in practically every adult *Plethodon glutinosus* male killed during the summer months. It will be found in a majority of *Gyrinophilus* males, but in only a small percentage of *Eurycea* and *Plethodon cinerius*. Its length varies, even in males of the same species, equal in size, killed at the same time of the year. It may be so short as to be scarcely distinguishable by gross examination, or it may approximate a fourth of the total length of the testis. Its diameter, usually rather uniform throughout, is but a fifth to a third that of the testis immediately anterior to it. The two regions, as a rule, are sharply differentiated as to size, the caudal portion or appendage not increasing perceptibly in diameter towards its junction with the larger "body" of the testis. (The two regions, indeed, may even be separated by a slight constriction.) The caudal appendage would usually differ in appearance, then, from the gradually tapering anterior end of the testis of figs. 1 to 7. It would differ, too, from the similarly tapering posterior end of the testis in *Ambystoma*, or the bluntly-rounded termination of the organ in other Urodele males lacking an appendage.

To understand the origin and significance of the appendage it is necessary to note its seasonal changes, both in gross appearance and in histological detail. These will be first outlined as they occur in *Plethodon glutinosus*, a species in which the appendage is practically a constant feature in the adult male. It is most conspicuous in animals killed from May to July. Since the body of the testis is then approaching its maximum diameter, due to



Figs. 1 to 7. A series of diagrams to illustrate various forms of the testis commonly encountered in Urodeles. The cephalic end of the testis is uppermost in each case.

Fig. 1. Elongated, cylindrical type of testis, tapering anteriorly, bluntly rounded posteriorly. Found in the Plethodons, *Eurycea*, and *Gyrinophilus* during winter months, and, in some males of these species, in summer.

Fig. 2. A modification of the testis of Fig. 1 resulting from earlier emptying of the caudal lobules. The anterior lobules still contain spermatozoa. A testis of this form is encountered during the fall or early winter months in the Plethodons and other Urodeles, and is reduced to the type of Fig. 1 by the final emptying of the anterior lobules.

Fig. 3. A modification of the testis of Fig. 1 resulting from earlier development of the germ cells in caudal lobules. Seen as a rule in spring months or in young males in which a smaller proportion of the lobules produce spermatozoa.

Fig. 4. Testis with a caudal reduction ("caudal appendage") resulting from degeneration of germ cells in the caudal lobules of the organ. Found commonly in *Plethodon glutinosus* during the summer months; less common in *Eurycea* and *Plethodon cinerius*.

Fig. 5. Testis with a constriction resulting from localized germ-cell degeneration. Found only occasionally, sometimes as an apparent preliminary condition to that illustrated in Fig. 4.

Fig. 6. "Multiple testis" of *Desmognathus* resulting from delayed regeneration of lobules emptied in each sexual cycle. Spermatozoa for the following cycle are always produced in lobules ahead of those maturing germ cells in the current season.

Fig. 7. Testis of *Gyrinophilus* in summer. In this species the caudal appendage, though structurally similar to that of *Plethodon glutinosus* (Fig. 4) is rendered less conspicuous by the greater length and reduced diameter of the organ as a whole.

the growth and divisions of its spermatocytes, the slender caudal appendage is naturally in obvious contrast as to size (Fig. 4). In the fall, after extrusion of the spermatozoa, the testis proper is greatly reduced in diameter; the appendage, meanwhile, has become considerably enlarged. During the winter, as a result, the testis appears of more nearly uniform diameter throughout, as in Fig. 1, with little or no indication of the caudal reduction so striking in the preceding summer. Nevertheless, in the spring the appendage once more makes its appearance.

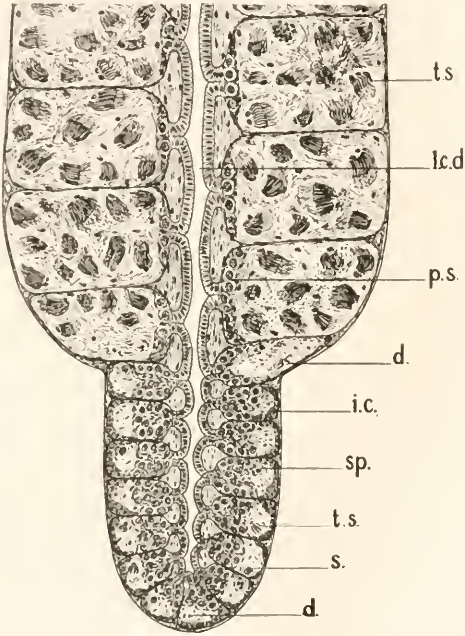


FIG. 8. Caudal third of the testis of *Plethodon glutinosus* in late June. Semidiagrammatic. The reduction in size of the lobules of the caudal appendage is strikingly shown; but little debris from degenerating cells now remains. Though the body of the testis at this season contains transforming spermatids, the lobules of the appendage show only occasional germ cells other than their apical spermatogonia. *T.s.*, transforming spermatids; *l.c.d.*, longitudinal collecting duct, central in position, surrounded by connective tissue; *p.s.*, primary spermatogonium (residual spermatogonium) of lobule apex; *d.*, nuclear débris from recently degenerating germ cells; *i.c.*, interstitial cells; *sp.*, group of spermatids; *s.*, a primary spermatocyte.

Examined in sections, the appendage of a male killed in early June is found to be traversed by the central collecting duct of the testis (Fig. 8), around which are lobules, radially arranged as in the testis proper, but of much smaller size. These small lobules contain, at their apices, primary spermatogonia of the usual type. The peripheral portions of the lobules are filled with cells of various types: a few secondary spermatogonia, an occasional growing or dividing spermatocyte, or small group of spermatids, and degenerating germ cells or cell debris among scattered Sertoli cells (see Figs. 8 and 9). The lobules of the testis proper contain, at the same time of the year, transforming spermatids, dividing spermatocytes, growing spermatocytes, and spermatogonia, in the caudo-cephalic succession characteristic of this and other Urodeles. In accordance with the usual postero-anterior development of the germ cells in these species (spermatogenetic

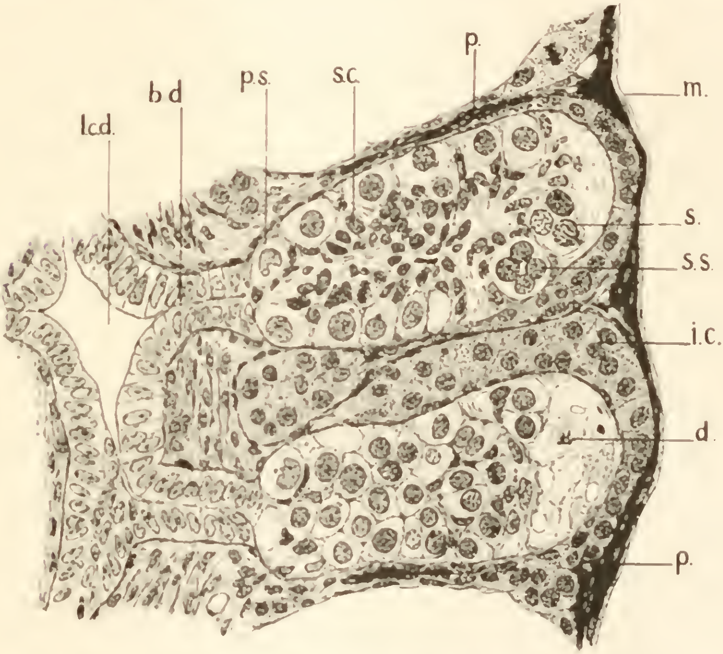


FIG. 9. Two lobules of the caudal appendage of *Plethodon glutinosus* in late June. Camera lucida sketch to show contents of lobule in greater detail. *L.c.d.*, longitudinal collecting duct; *b.d.*, branch of longitudinal duct leading to lobule; *p.s.*, primary spermatogonium of lobule apex; *s.c.*, Sertoli cell (follicle cell); *p.*, pigment cells of tunic and septa; *m.*, mesothelial covering of testis; *s.*, spermatocyte I; *s.s.*, secondary spermatogonium; *i.c.*, interstitial cell sheath around lobule; *d.*, degenerating cells or débris from same.

wave; Kingsbury, '02; Humphrey, '21, '22), the caudal region or appendage would be expected to contain at this time, in addition to its residual primary spermatogonia, only cells further advanced in development than those of the region anterior to it. That is, we should expect to find here only numerous spermatids undergoing transformation, rather than the several sparsely-represented cell types actually encountered.

Later in the season (July, August) it will be found that some of the spermatids in the appendage may have undergone transformation; a very small number of spermatozoa may eventually mature and leave the lobule. This number is as a rule negligible. The slow increase of secondary spermatogonia is the most important change in the lobule of the appendage during the summer; these arise by proliferation of the residual primary spermatogonia of the lobule apex. Such primary spermatogonia always are to be found in the appendage, though all other germ cell stages be absent. The appendage is therefore not a sterile region of the gonad, as is the caudal portion of the testis of certain Teleosts. Germ cells are never entirely lacking.

After the extrusion of the spermatozoa from the testis proper, a prompt regeneration of its emptied lobules is accomplished through the rapid multiplication of their apical spermatogonia. This, indeed, begins some time before the lobules are actually emptied. The development of spermatogonia in the lobules of the caudal appendage now keeps pace with—or, to be exact, precedes—their development in the lobules directly anterior to it. The enlargement of its lobules, of course, explains the disappearance of the appendage as such in animals examined in autumn, the division of the testis into two regions becoming more and more completely obliterated, as lobules throughout the testis become uniformly filled with secondary spermatogonia.

The spermatogonia of the regenerated lobules now begin to undergo the characteristic transformation by which they become spermatocytes of the first order. The latter then enter upon their growth period. Following the rule of progressive caudocephalic development, the formation of spermatocytes first occurs in the most caudal lobules, formerly the territory of the appendage. Lobules successively farther and farther cephalad proceed in development after the same manner, until in October from half to three fourths of the testis contains primary spermatocytes. Meantime, however, in the most caudal lobules these spermatocytes have completed their maturation divisions. Since these too proceed in regular caudocephalic order, it follows that the most caudal portion of the testis finally will contain spermatids. These, in *Plethodon* males killed in late October or in November may be found in as much as a fourth of the extent of the testis, or, on the other hand, in but a few of its most caudal lobules. The onset of cold weather finally checks the further course of spermatogenesis, and few or no maturation divisions then occur until the following spring.

It is in the fate of those spermatids formed in autumn that we find the correct explanation of the caudal appendage seen in the following May or June. For these spermatids, apparently unable to transform, undergo degeneration and resorption, disappearing during the early spring months. The lobules which they occupied now become very much reduced in size, and the caudal portion of the testis, as a result, assumes the proportions of a degenerate appendage of the larger anterior part. In this appendage, as has been mentioned, an occasional spermatocyte may develop (See Fig. 9) and go through its maturation divisions; this may sometimes result in the belated formation of a very small number of spermatozoa. Any extensive development of spermatogonia and spermatocytes in this region, however, is delayed until the time when such a development would occur in preparation for the next spermatogenetic cycle. It is not until late summer or early fall, therefore, that the growth processes in the appendage, now equivalent to those of the lobules anterior to it, obliterate the distinction between the two regions.

A caudal appendage similar to that in *Plethodon glutinosus* is found in practically all *Gyrinophilus* males killed during the summer months. In this species, however, the greater length and reduced diameter of the whole organ (Fig. 7) renders the appendage less conspicuous. Microscopically its structure is comparable to that of the appendage in *Plethodon*, although it shows an apparently greater effort toward compensatory development of germ cells. All the reduced lobules contain a small number of primary spermatocytes, or, later in the season, spermatids or spermatozoa. Because of their small number, these germ cells do not interfere with the increase of spermatogonia at the central apex of the lobule. In early fall, consequently, spermatogonia are more abundant and farther advanced in development here than in the body of the testis. With the extrusion of the spermatozoa from the latter region, and its consequent reduction in size, the distinction between body and appendage is obliterated, as in *Plethodon*.

The caudal part of the testis, in October and November, always contains spermatocytes in the growth period. Just as in *Plethodon* the germ cells of this region precede in development those of more anterior lobules. In *Gyrinophilus*, however, the spermatocytes do not begin their maturation divisions in the fall. No spermatids appear in a specimen killed on November 19. In *Plethodon glutinosus* the caudal fourth of the testis may contain spermatids as early as November 1.

How, then, does the appendage of *Gyrinophilus* arise? No spermatids are formed in autumn, to degenerate during the winter as in *Plethodon*. Nevertheless the appendage is of almost as constant occurrence in one species as in the other. A clue to its origin in Gyrinophilus is furnished by specimens killed in late March. In these the testis is already greatly reduced in diameter at its posterior end. Here the lobules contain, in addition to their apical spermatogonia, only a very few primary spermatocytes. The majority of these latter cells have completely disappeared; the peripheral portions of the lobules they occupied are now greatly reduced in size and contain only a syncytium of Sertoli cells. Toward the body of the testis, however, the diameter of the appendage increases. Lobules in this region show within the syncytium of Sertoli cells, traces of cell debris and cells far advanced in degeneration. Still farther forward, in lobules of approximately the normal size, the earlier stages of this cell degeneration are to be encountered. Here one finds all intermediate conditions between the small, densely-staining masses characteristic of karyolytic cells in advanced stages, and primary spermatocytes in which the first indication of degeneration is to be seen in a slight contraction of the nucleus and a massing of its chromosomes. Degeneration seems to begin when the spermatocytes are in the midst of their growth period, with the chromosomes already well-defined and definitely oriented with respect to the centrosomes. In none of the animals killed in either November or March were maturation divisions to be encountered.

It would appear, therefore, that in *Gyrinophilus* a degeneration of primary spermatocytes takes place during the winter months. Since cells of this stage of development are found in autumn only in the caudal part of the testis, it follows that only this region will be reduced in size through their later degeneration and resorption.

In *Eurycea* and in *Plethodon cinerius* the caudal appendage is much less frequently encountered than in *Gyrinophilus* or *Plethodon glutinosus*. In these species, too, it is frequently so small as to be overlooked, except in serial sections of the organ, and, in many cases, is so poorly marked off from the body of the testis that it appears to be merely a caudal tapering of that structure. In its microscopic structure, and in its mode of development it is, however, entirely comparable to the appendage in the forms already considered.

In *Eurycea* males a considerable proliferation of germ cells in the appendage often causes it to resemble a testis in miniature, with the same seriation of developmental stages as occurs in the body of the testis ahead of it. Its germ cells are always retarded in their development, however, as compared with those of the testis proper. For example, in a well-defined appendage of this sort, in mid-August, the caudal lobules contain untransformed spermatids, while the corresponding caudal lobules in the body of the testis are filled with well-developed spermatozoa. Earlier, in July, I have found an appendage filled with spermatocytes I, the body of the testis at the same time containing transforming spermatids throughout its caudal half.

The mode of origin of the appendage in *Eurycea* differs only in details from that described for other species. The primary spermatocytes do not normally develop until the spring months. A male killed in early May, for example, has the caudal third of the testis filled with the so-called synizesis figures characteristic of the early part of their growth period. The development of a caudal appendage through a winter or spring degeneration of spermatids (or growing spermatocytes) is impossible in such an animal. The caudal lobules of the testis in spring show the probable mode of development of the appendage in *Eurycea*. These lobules contain numerous karyolytic cells, particularly in their peripheral portions. In the most caudal lobules, a syncytium of Sertoli cells, with cell debris and fat droplets in its meshes, indicates the region of earliest degeneration; here the lobules are already greatly reduced in size. More anteriorly fewer degenerations have been completed and the earlier stages of nuclear breakdown are readily observed. Here the lobules are as yet but little reduced in size. An appendage of the testis is therefore not sharply marked off at this time, the entire posterior third of the testis having the form of a short cone.

The germ cells appear to begin degeneration immediately after synizesis, or possibly to degenerate without recovery from this condition. They are stricken, therefore, at the very beginning of the spermatocyte growth period, rather than after it has well started, as in *Gyrinophilus*.

It is worthy of note that the proliferation of spermatogonia to replace degenerated germ cells seems to begin early in May. Since a similar proliferation of spermatogonia is taking place at this same time in the more anterior lobules of the testis, it follows that the appendage, later in the year, will correspond in development with the more anterior rather than the posterior portion of the organ.

The appendage of the testis in *Plethodon cinerius* is well developed in but relatively few males. Frequently it includes only two or three caudal lobules; in such cases it would of course be passed over unobserved in gross examination. The structural features of such lobules, however, warrant classing them with the well-developed appendages of other males.

The development of the appendage may be briefly outlined. In males killed in November or December there has been as yet no development of spermatocytes or spermatids. Yet in animals killed as early as March a caudal appendage may be present. At this time its anterior lobules show karyolytic cells; in the body of the testis immediately adjacent (or even in the same lobule or the same cyst with degenerating cells) are spermatogonia of the last generation, ready to transform into spermatocytes. The germ cells therefore appear to be stricken and degenerate during that critical period of transition from spermatogonium to spermatocyte. In such a period, also, occur the degenerationsdescribed by Kingsbury and Hirsh ('12) in *Desmognathus*. These, however, take place at the end of the spermatogenetic cycle, checking the further development of primary spermatocytes for the season, while the degenerations just described in *Plethodon cinerius* occur at the beginning of the cycle and delay the development of spermatocytes until a later (and presumably more favorable) period. By May the caudal portion of the testis proper contains normal growing spermatocytes, while traces of degenerating cells in the appendage have practically or entirely disappeared. During the remainder of the summer the lobules of the appendage contain only a limited number of spermatogonia. There appears to be no compensatory development of spermatocytes as in *Eurycea* or *Gyrinophilus*, so that no germ cells at all are matured in the appendage. In this respect *Plethodon cinerius* is similar to *Plethodon glutinosus*, in which few if any germ cells come to maturity in the appendage. Its spermatogonia proliferate during the summer and early fall. Their increase in number, and the extrusion of the spermatozoa from the body of the testis, at length obliterate the size difference between the two regions, as in the other species considered.

The appendage of the testis in *Plethodon* and other Urodeles, as above described, is clearly different, both in origin and fate, from the reduced caudal portion of the testis often seen in *Desmognathus* and *Diemyctylus*. The latter structure, in adult males, arises by the emptying of the entire region producing spermatozoa. This, of course, occurs at the end of the spermatogenetic cycle. The emptied lobules are not at once restored by proliferation of their residual primary spermatogonia, which in these species may remain quiescent for several months. The result is the reduction of the emptied portion of the testis to a slender cord. Regeneration of the lobules finally taking place at the caudal end of this cord, an enlargement arises at that point, separated from the testis ahead of it by a slender intervening region in which lobule regeneration has not yet occurred (Fig. 6). In this manner arises the so-called lobed or multiple testis of *Desmognathus* and *Diemyctylus* (Humphrey, '22). A necessary condition for its appearance is delayed lobule regeneration. In the species possessing an appendage, quite the reverse of this condition obtains. Lobule regeneration proceeds without delay, beginning even before the spermatozoa have left the testis. The spermatogonia of the caudal lobules may even transform to spermatocytes, and these complete their growth and maturation periods, within a few weeks after emptying of the lobules begins. It is this precocity of development, in fact, which results in the formation of a caudal appendage, since cells reaching a certain stage during the fall and winter are fated to degenerate. Though this reduces in size the caudal region of the testis which these germ cells occupied, such reduction is never so extreme as is that of the emptied testis of *Desmognathus*, nor is its regeneration so long delayed. The appendage of *Plethodon*, for example, is always restored to the normal diameter of the testis by the proliferation during the summer of spermatogonia for the next sexual cycle; the emptied region in *Desmognathus* does not begin regeneration for several months longer, and even then, at first, only at its caudal extremity. A part of the emptied region in *Desmognathus* or *Diemyctylus* thus comes to be a slender intermediate cord joining two lobes of a multiple testis. The appendage of *Plethodon*, on the other hand, is annually restored to the normal diameter of the testis proper, and a multiple testis in this species is unknown.

From the preceding outline of its origin and fate in different species, the variations in length and diameter of the true appendage should be easily understood. Its length depends upon the number of caudal lobules in which germ cell degeneration has occurred; its diameter varies with the degree to which the products of degeneration have been resorbed, or the extent of the restoration of its germ cells by proliferation of the residual spermatogonia. Its occasional complete absence in summer months indicates that none of the usual degeneration has occurred in the preceding seasons; its constant absence in fall and winter months is due to the annual development of spermatogonia, a process which is common to both the appendage and the testis proper. This obliterates the structural differences between the two regions, leaving them alike save for the slightly more advanced condition of the germ cells in the territory of the appendage.

In addition to its variations as between species and between individuals of the same species, the appendage undoubtedly varies in length in the same individual in successive years of its adult life. Environmental conditions differ from year to year; this variation is likely to be reflected in the spermatogenetic processes. If, for example, the climatic conditions of autumn favorable for the maturation divisions of the spermatocytes (in *Plethodon glutinosus*) are continued long beyond their usual period, a greater portion of the testis is filled with spermatids. If, on the other hand, these maturation divisions are checked by the unusually early onset of cold weather, fewer caudal lobules will begin the winter containing spermatids. In the former case, the appendage will be longer, and in the latter case, shorter, when the degeneration of these spermatids has been completed. In some animals, of course, few or no germ cells may reach the critical period of development in the fall months; in this, probably, lies the explanation of the absence of an appendage—a condition frequent in *Eurycea* and *Plethodon cinerius*, though rare in *Gyrinophilus* and *Plethodon glutinosus*. In *Necturus* and *Cryptobranchus* a caudal appendage never develops; a seasonal degeneration of germ cells, of it occurs in these species, is either not limited to one region of the testis or is not sufficiently marked to cause a noticeable reduction in its size.

Degeneration of germ cells in the Urodele testis was early described by Flemming ('87) in *Salamandra*. These cells, it appears, were secondary spermatogonia. From the descriptions of Flemming it would seem that they were not definitely localized in the spermatogenetic cycle or limited to a definite region in the testis. Hermann ('88) describes these same degenerations in greater detail; neither he nor Flemming advance suggestions as to their cause or significance. Drüner ('94) later attempted to demonstrate that these degenerations were caused by the action of a parasite upon the germ-cell nucleus.

The work of Kingsbury and Hirsh ('12) first called attention to the occurrence of such degenerations at a definite point in the spermatogenetic cycle of *Desmognathus*. In this Urodele, the secondary spermatogonia of the "last generation" in each sexual cycle are apparently unable to complete their transformation to spermatocytes and begin the usual spermatocyte growth period. Instead, they undergo degeneration and ultimately disappear.

The polarity of the testis in *Desmognathus*, and the cephalo-caudal seriation of its germ cell stages results in the localization of the degenerating cells in a "zone" near the anterior end of the organ. Cephalad of this, as a "boundary plane," according to Kingsbury and Hirsh, the spermatogenetic processes now lag, no more spermatocytes developing until at the beginning of the next cycle. The degenerations, it is therefore suggested, bear some relation to a regulation of the spermatogenetic processes.

To the writer's knowledge no detailed study of boundary plane degenerations in other Urodeles has ever been reported. The occurrence in *Diemyctylus* of a "boundary plane" comparable to that of *Desmognathus* was noted briefly in a previous paper (Humphrey, '21). The degeneration of spermatogonia in these two species occurs at the same point in the sexual cycle and is limited to a corresponding region of the testis. Similar degenerations would doubtless occur at a comparable time and place in any Urodele showing the same general plan of spermatogenetic pattern (*i.e.*, slow spermatogenetic wave, with delayed lobule regeneration—features resulting in a multiple testis). Spermatogonial degenerations unquestionably occur in Urodeles with a different pattern of spermatogenesis, but in these they are not so definitely localized in the testis and do not affect entire lobules; hence no well-marked "plane" is established.

Though "boundary plane" and other spermatogonial degenerations are possibly associated with a regulation of the spermatogenetic process, it seems unlikely that the degenerations described in this paper have any such significance. They occur at the opposite end of the testis, and—so to speak—at the opposite end of the spermatogenetic cycle. The cells that suffer degeneration are not the last generation of a season's output, but the first. They in each case are most advanced in development of any cells in the testis at the time. They are precociously developed—in some cases several months ahead of the similar cells privileged to go on to complete maturity. Spermatids formed in May in *Plethodon glutinosus*, for example, transform into functional spermatozoa, but spermatids developed in the preceding November usually undergo degeneration before spring. Winter, in this case, comes on after the beginning of a new spermatogenetic cycle; certain cells apparently have reached a point in development beyond which, under the now unfavorable conditions, further progressive changes seem inhibited. Nor can these cells remain in a resting condition until the advent of spring. Their degeneration and resorption from the caudal lobules of the testis follows, with the results already pointed out.

Strangely enough, the point in development at which the cells are stricken varies with the species. There is, apparently, no one "critical period" common to the four species studied. The four agree in that the germ cells suffering degeneration are in each case those most advanced in development, located in the most caudal lobules of the testis. In *Plethodon glutinosus* these cells are spermatids. Spermatocytes in their growth period are not affected. Yet it is in this latter stage that the germ cells of *Gyrinophilus* begin degeneration. Rather curiously, those germ cells of both *Gyrinophilus* and *Plethodon* which chance to be transforming from spermatogonia to spermatocytes remain unaffected. This transformation period, it will be recalled, is that in which the "boundary plane" degenerations always begin. It is the cells of this stage of development which degenerate in *Eurycea* and *Plethodon cinerius* to produce the caudal appendage. The absence of such an appendage in numerous males of these two species might be assumed to be due either to an absence of cells at the critical stage, or to an increased ability of these cells to survive unfavorable conditions. That the appendage is seldom absent in *Gyrinophilus* and *Plethodon glutinosus* is evidence of the constancy with which germ cells reaching more advanced stages degenerate.

So far as may be perceived, there is nothing inherent in their caudal position in the testis which should cause germ cells to degenerate. This region is apparently as well vascularized as any portion of the testis; its germ cells in autumn show no indication of interference with their development, being always somewhat in advance of those more anteriorly situated. Nor do germ cells of *all* stages degenerate here. The primary spermatogonia always remain unaffected, and later restore the lobules of the appendage through their proliferation. The degeneration, in short, is clearly correlated with the more advanced stage of development of the cells involved, only these cells being unable to survive when conditions become unfavorable. Such unfavorable conditions, of course, are imposed upon all cells of the testis alike, without reference to their position in the organ.

That cells in more advanced stages of spermatogenesis are least able to withstand unfavorable conditions has been demonstrated experimentally in other animals. Siperstein ('21), for example, has found that in adult white rats subjected to inanition, the more advanced stages of the germ cells are the first to undergo degeneration. Moore ('24), in his study of testis transplants recovered after one to seven months, points out that some influence "prevents the building up of a completed epithelium with differentiated spermatozoa; instead the cells near the stage of differentiation either degenerate in place or are cast off into the lumen of the tubule." In his report on the changes in testes rendered cryptorchid by operation, Moore further states that spermatozoa rarely are present in the tubules seven days after operation; later the remaining cells of the germinal epithelium disappear, the cells persisting longest being those at the periphery of the tubules: *i.e.*, the spermatogonia. It would appear, therefore, that in mammals, as in amphibia, the cells first affected by departure from the normal conditions are those most advanced in development. In the four Urodeles discussed in the preceding pages, conditions become but slightly unfavorable, and only certain of the most advanced cells are affected. A condition comparable to that obtained by Moore in mammals, may, however, be obtained in Urodeles by subjecting the males to inanition over a considerable period of time. The entire testis, in such animals, may be reduced to a slender cord in which no germ cells save primary spermatogonia remain. The lobules as such may entirely disappear. This condition is somewhat comparable to the extreme reduction of the mammalian testis in cryptorchidism, save that in the mammal the reduced tubules are persistent.

In no Urodele examined does germ-cell degeneration commonly reduce more than a fourth of the testis to an appendage. One of even this proportionate length is unusual. It is clear that animals with an excessive degeneration of germ cells would produce fewer mature spermatozoa than males not showing this peculiarity. Such males would probably leave fewer offspring than the male producing a greater number of functional germ cells. Hence an appendage of excessive length would tend to disappear in the course of phylogeny, the strains surviving being those in which only a more limited degeneration occurs.

In one male however, an unusual type of appendage, fully half the length of the testis has been noted. This male, a *Gyrinophilus* freshly captured on August 15, is of large size, and is possibly a senile individual or one unfavorably affected by disease or other factors. At any rate, the spermatids in the entire caudal half of the testis have failed to transform, degenerating as they developed. This degeneration, it may be noted, is of cells more advanced in development than those whose degeneration in winter ordinarily gives rise to the appendage in this species. The growing spermatocytes, though unable to survive winter conditions, actually complete their growth period and maturation divisions in this specimen, only to have the resulting spermatids destroyed by subsequent degeneration.

Another unusual type of appendage was found in a *Eurycea* male captured in early July and kept in the laboratory for a period of about ten days. The oldest spermatids in this male were probably beginning their transformation at the time of its capture, since, ten days later, developing spermatozoa were present in a few of the most caudal lobules of the testis. Apparently only those spermatids having reached a certain stage of transformation before the period of captivity began were able to continue their normal differentiation, since in lobules more anterior in position, in which the spermatids would have been less advanced, their continued development had become abnormal in character. In these lobules, as a result, various bizarre types of spermatozoa were present, including cells similar in form to the mammalian spermatozoön. In still more anterior lobules the younger spermatids had disappeared completely, probably without ever beginning transformation; cephalad of this region were lobules in which the spermatid degeneration was still going on. Younger stages (spermatogonia, growing and dividing spermatocytes) show no unusual features. Apparently those germ cells most susceptible to environmental effect are those most advanced in development—at least up to a certain stage in spermatid transformation. After this stage the cells are more resistant, as evidenced by the normal spermatozoa in the most caudal lobules of this male. Fully mature spermatozoa, of course, would probably be even less susceptible than those in transformation. Their survival of the winter in the testes of numerous Urodeles (*Diemyctylus*, *Necturus*, *Ambystoma*, etc.) would bear out this hypothesis.

Unusual types of appendage, such as the two above discussed, further indicate that the similar structure found under usual or normal conditions is but a result of the action of environmental factors upon the spermatogenetic processes. The caudal appendage of such general occurrence is no indication of a functional reduction of the testis in phylogeny or ontogeny, nor does it represent a structural adaptation for a changed function, as do sterile portions of the gonads in certain Teleosts. Its occurrence is limited to testes of a simple type, in which the germ-cell stages occur in caudo-cephalic succession, and cells of any one region are all in approximately the same stage of development. Under such conditions, germ-cell degeneration limited to cells of a particular type readily affects the form of the testis in a particular limited region. In a testis in which every tubule contains cells of numerous types (as in anuran, reptile, bird, mammal) such modifications of the organ are of course impossible.

Summary.

1. The testes of several species of Urodeles frequently possess a slender caudal extension or appendage suggestive of a functional reduction of the organ.

2. This caudal appendage is present only during the summer months. It disappears in autumn, to reappear in the following spring.

3. Study of its cyclic changes indicates that its appearance is due to degeneration of the germ cells of the more caudal lobules of the testis.

4. The germ-cell degeneration is interpreted as resulting from the unfavorable conditions of winter; the cells most advanced in development are apparently the most susceptible and they alone degenerate.

5. The caudo-cephalic order of development of the germ cells results in the localization of the most advanced stages in the caudal end of the testis; their degeneration reduces this region to a slender caudal appendage.

6. The development of spermatogonia for the next spermatogenetic cycle occurs at the normal time in the appendage, increasing its size to that of the "body" of the testis: this explains the annual disappearance of the appendage in species possessing it.

7. The development of an appendage of the type here described is possible only in certain Urodeles with a testis of simple type and marked polarity. It is of almost constant occurrence in *Gyrinophilus* and *Plethodon glutinosus*.[1]

Postscript.—Since the foregoing paper was placed in the hands of the editor, a number of Urodele males captured in the spring and summer of 1924 have been examined. These animals, particularly in the case of one species, show a very striking difference from those of the four preceding years. While among twenty-two adult *Plethodon glutinosus* males captured between January and September in 1920, '21, '22, and '23, only one lacks the characteristic appendage above described, not one of the nine taken in 1924 possesses this structure. In those males captured earliest in the season (May and early June, 1924), normal spermatocytes still occupy that caudal portion of the testis in which spermatids are commonly found to have been formed in early autumn. Evidently few or no maturation divisions occurred in the fall of 1923, and as a result, few or no spermatids were present to degenerate during the winter of 1923–24. Absence of the appendage in 1924 is the natural result of such retarded spermatogenesis.

The failure of the maturation divisions to begin in the fall of 1923 is undoubtedly a consequence of climatic conditions obtaining during the spring and possibly also the fall of that year. Weather bureau records at Ithaca show that the average temperature for May, 1923, was 4.8 degrees below that of the normal (average of 30 years). *Plethodon* males taken in July, 1923, show spermatogenesis retarded by from two to four weeks as compared with males taken in other years,—a retardation presumably due to the lowered temperature above noted. This retardation apparent in July would undoubtedly delay completion of the current spermatogenetic cycle and postpone the beginning of the cycle following. At any rate, no maturation divisions occurred in the fall of 1923 in this species. The spermatocytes present in the testis were either delayed in their early development, or their maturation divisions were later prevented by lowered October temperature. Both factors might conceivably be involved in effecting the end result, the elimination of the usual autumnal maturation. Since only spermatids undergo degeneration during winter in *Plethodon glutinosus*, no appendage appeared in the following season.

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[1] The writer wishes to acknowledge the helpful suggestions and criticism of Dr. B. F. Kingsbury of Cornell University, in whose laboratory the investigation was begun.

Of the other Urodele species developing an appendage, *Plethodon cinerius* shows an effect comparable to that in *Plethodon glutinosus*. In *Eurycea* the appendage was found during 1924 much as in preceding years. No *Gyrinophilus* males were examined.

The complete absence of the appendage here reported strikingly supports the writer's statement that it is a structure due to environmental effects upon spermatogenetic processes, and as such, may vary from year to year even in the same male.

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AN APTEROUS MUTATION IN *BRUCHUS*.[1]

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There appeared in my cultures of *Bruchus quadrimaculatus* Fabr., the "four-spotted cowpea-weevil," a mutation entirely void of wings. It was discovered at Cold Spring Harbor on August 5, 1918, in seven females. The wild stocks from which these wingless females emerged had passed through twenty-five generations of selective breeding. This mutation could have only come from a single pair. The culture was obtained from Raleigh, North Carolina, on January 18, 1916, and had been examined daily for various mutations. It is known therefore that at no previous time had any such apterous beetles appeared. During the eight years of breeding this insect no more such mutations have been observed among the thousands of weevils examined. The appearance of this Bruchid's wings is not unlike that described by Metz ('14) for apterous *Drosophila*. Even though the cultures of this apterous insect died out, due possibly to some genital defect, there are some genetic interpretations of value which may be made.

This apterous beetle was first discovered in seven females which made their appearance during two days from one wild culture bottle. Because they were the first mutants observed in such great numbers, they were at first thought considered abnormalities or freaks rather than mutations. But when they again emerged in the F_2 from apterous females mated with wild males from another North Carolina culture, it was evident that this trait was a recessive one.

Concerning the origin of mutations in *Drosophila* Lancefield ('18) states that if a mutation occurred in the early oögonial stages several with the new gene should appear. This answers the question why several of the mutants could have been revealed. The fact that they were all of one sex might add evidence to such a conclusion. However, in the tests which follow, it is demonstrated that the genes are carried by both sexes, but are visible only as phenotypes in the females. The trait is therefore sex limited. If this is true, the character could have originated possibly in the male previously but remained unseen. A recessive mutation, like apterous, could not be seen in either sex until the second generation after its mutation. This is the probable origin and accounts for the seven apterous insects discovered in the same generation.

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[1] Contribution from the Zoölogical Laboratory of the University of Oklahoma, Second Series, No. 46.

Each apterous *Bruchus* was mated to a normal winged brother from a second North Carolina culture. Seven pairs in all were mated (Table I.), but only two produced progeny, pairs 2 and 6. Pair 2 in the F_1 generation produced 4 normal winged females and 5 normal winged males. These were inbred as a mass culture in the F_2, giving 13 winged females, 2 apterous females, and 11 winged males. The two apterous females were mated with an F_2 winged male from this same fraternity but no offspring were found. Pair 6 was the other of the seven apterous insects which had progeny. They produced in the F_1 9 winged females and 8 winged males. These F_1 beetles were inbred as a mass culture. Their progeny consisted of 9 winged females, 3 apterous females, and 12 winged males in the F_2. Each of these three F_2 apterous beetles was mated with a male from the same fraternity, but no progeny was found from any pair of the three. Again from this same F_2 fraternity 6 winged females were bred to five winged males. They produced in the F_3 7 winged females 3 apterous females, and 9 winged males. Another winged pair from the F_2 gave five winged females and two winged males in the F_3. The last two of the F_2 females were mated with the last 5 winged males of this same fraternity. They produced in the F_3 6 winged females, 4 apterous females, and 12 winged males. Each of these four, F_3, apterous was bred with a winged male from a wild culture, the same North Carolina stock but only two pairs gave offspring in a mass culture consisting of 8 winged females and 7 winged males in the F_1 generation. These bred in mass produced 25 winged females, 4 apterous females, and 30 winged males. This apterous trait came to an end when each of these four wingless females were each mated with a male from a North Carolina culture from the experiment table. The apterous insects could possibly have been saved by entirely mass cultures, crossed with stocks having low sterility or high fecundity.

This rather incomplete data shows that apterous is a simple recessive. That the majority of apterous females did not produce offspring might be correlated with the idea that such females have genital openings too small for the male copulatory organ to enter. Another apparent fact is that sterility was manifested throughout, which might be interpreted as being due to the stock from North Carolina in which apterous originated and in which all apterous matings were made.

The wild North Carolina cultures, in which the apterous insects were discovered, were dead by November. They always produced a less number of progeny than any other wild stock since sterile pairs were always common; however, the Texas wild cultures continually produced a great number of offspring. All wild stocks were kept from generation to generation by mating one or five pairs each time. More pairs mated possibly would have been sufficient to have preserved the North Carolina stocks alive. This sterility could have been eliminated by crossing apterous to the Texas cultures instead of the one in which it arose, but was the only one present at this time. This relative infecundity, further, accounts for the low number of offspring discovered in these experiments. It is unknown to which autosome apterous or sterility was linked, because no crossover tests were possible.

The average number of progeny produced per pair is the lowest observed. Table I. for 50 pairs mated gives 198 offspring, which is for each pair an approximate 1:4 ratio. For a comparative conception the following ratios are summarized from another set of experiments: a 1:5 ratio for pure lines of white body color and black spots on elytra, the $R^w s$ cultures; a 1:38 ratio for red body color and black spots on the elytra, the Rs cultures; a 1:50 ratio for red body color and red spots on elytra, the RS cultures; a 1:46 ratio for black body color and black spots on elytra, the $R^b s$ culture; and, lastly, one wild culture, rs, gave a 1:52 ratio. This wild culture is the highest of all in giving a greater average of adults from each pair bred.

It is about thirteen times more prolific than the apterous wild stock from North Carolina. Aside from this extremely low number of progeny manifested in apterous matings, it is very probable that there must be some defect of the genital organs of the apterous females because (Table I.) of the nineteen pairs of apterous females mated only four produced offspring. Two of these were single pairs of the first seven apterous mutations: Pairs 2 and 6 respectively. The other two apterous pairs were bred as mass cultures and they produced offspring. Aside from this apparent sterility, it is possible that the genitalia of the female is too small for the male to copulate with the female in every instance.

TABLE I.

COMPLETE BREEDING RECORDS FOR APTEROUS.

The F_1 off-spring are indicated below as all winged with no apterous.
The F_2 progeny show both winged and apterous.

Mating No.	Pairs Mated Apterous Females with Winged Males.	Offspring.			Total.
		Females.		Males, Winged.	
		Winged.	Apterous.		
1	1	0	0	0	0
3	1	0	0	0	0
4	1	0	0	0	0
5	1	0	0	0	0
7	1	0	0	0	0
2	1	4	0	5	9
2.1	4*	13	2	11	26
2.11	1	0	0	0	0
2.12	1	0	0	0	0
6	1	9	0	8	17
6.1	9*	9	3	12	24
6.11	1	0	0	0	0
6.12	1	0	0	0	0
6.13	1	0	0	0	0
6.14	6*	7	3	9	19
6.15	1*	5	0	2	7
6.16	2*	6	4	12	22
6.161	1	0	0	0	0
6.162	1	0	0	0	0
6.163	2	8	0	7	15
6.1631	8*	25	4	30	59
6.16311	1	0	0	0	0
6.16312	1	0	0	0	0
6.16313	1	0	0	0	0
Totals	50	86	16	96	198

* Indicates all winged pairs in F_1 with F_2 offspring.

Metz ('14) states that low viability of the apterous flies causes more normals in the F_2. This is also true in *Bruchus* since there are more normals in the F_2, which consists of a total of 65 winged females, 16 apterous females, and 76 winged males. The ratio is approximately a 4:1 instead of a 3:1 ratio, because in such matings there would naturally be more pure dominants concerned. Aside from this the males do not show the trait which would also tend to make the ratios less normal.

Metz ('14) again, with reference to apterous flies being sex linked, states that the absence of apterous flies in the F_1 indicates that the character is not sex linked. It is true in *Bruchus* that the F_1 produces no apterous beetles, but this is not a safe criterion in *Bruchus*, because all males are always winged, regardless of their transmitting the apterous trait, that they may carry. The F_2 is the ideal place to determine it. All of the P_1 matings were apterous females with winged males. The F_2 if sex linked should give half females apterous and half winged or the reciprocal cross would give all females winged. This is not true for apterous in *Bruchus*, because the ratio approximates a 3:1 ratio in the F_2. The character is therefore not sex linked.

In conclusion, apterous is a recessive trait seen only in the females. It has a high degree of sterility possibly from the North Carolina stock in which it originated. Lastly, it has a lower viability than the normal.

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ASEXUAL REPRODUCTION IN THE STARFISH, *SCLERASTERIAS.*

W. K. FISHER.[1]

In sea stars, self-division involving the disk occurs much more rarely than autotomy of rays only. The first condition, termed fissiparity, is a form of asexual propagation and in the Asteriidæ is characteristic of three genera, namely, *Coscinasterias* (both the *calamaria* type and the *tenuispina* type), *Sclerasterias*, and *Stephanasterias*. In the last genus asexual reproduction appears to be fully as important as gametic. In *Linckia*, belonging to a different order, an entire animal may be regenerated from a cast-off ray. The curious comet forms originate in this manner.[2]

In *Coscinasterias* and *Stephanasterias* fissiparity persists in adult life, but in *Sclerasterias* [3] it is strictly confined to a very immature stage, in which the number of rays is predominantly *six*, whereas in the adult it is five. Furthermore these young differ so markedly from the adult [4] that they have been described as of a different genus. I have found this condition in three species: *Sclerasterias euplecta* (Fisher), *S. heteropœs*, new species, and *S. alexandri* (Ludwig). The young of the last was described by Ludwig as *Hydrasterias diomedeæ*, while *Hydrasterias richardi* (Perrier) is the fissiparous phase of an unknown adult.

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[1] From the Hopkins Marine Station of Stanford University.

[2] For figures see Fisher, "Asteroidea of the North Pacific," Bull. 76, U. S. Nat. Mus., 1911, plate 48, Figs. 1 and 2.

[3] *Sclerasterias euplecta* is figured in Fisher, "The Starfishes of the Hawaiian Islands," U. S. Fish. Comm. Bull. for 1903, part 3, plate 42, Figs. 1 to 4. Figure 3 is in the intermediate stage. The closely related S. *hypacantha* is figured in Fisher, "Starfishes of the Philippine Seas," Bull. 100, U. S. Nat. Mus., 1919, pl. 141, Figs. 2, 3.

The species of *Sclerasterias* resemble *Marthasterias glacialis*, the well-known European sea star. The genus is a fairly homogeneous group of thirteen known species, related to *Coscinasterias*. Its type, *S. guernei*, was supposed to be a highly peculiar small species but proves on examination to be immature and congeneric with *Eustolasterias* Fisher, a name of later application. From among the thirteen species may be mentioned: *S. alexandri* (Ludwig), Bay of Panama; *S. contorta* (Perrier), Florida to Barbados; *S. euplecta* (Fisher), Hawaiian Islands; *S. eustyla* (Sladen), Tristan da Cunha; *S. guernei* Perrier, genotype, Bay of Biscay; *S. mollis* (Hutton), New Zealand; *S. tanneri* (Verrill), Atlantic coast of United States; *S. heteropæs* Fisher, new species, Monterey Bay, California.

Perrier states that his genus *Lytaster* is fissiparous. I can confirm this from an examination of the types, at Paris. But the types are only very immature specimens of *Coscinasterias tenuispina*.

For a synopsis of the family Asteriidæ, see Fisher, *Annals and Mag. of Nat. Hist.* (9), Vol. 12, 1923, pp. 247, 595.

In this stage the sea stars actively divide by splitting into equal halves. The number of these divisions is not known. In one instance, in *S. euplecta*, a division has taken place nearly at right angles to the plane of the prior fission. Since adult *Sclerasterias* are almost invariably five-rayed and the majority of the young are six-rayed, an arm is lost somewhere in the process—probably at the last division. These dividing young range in size from R 8 mm.[5] to R 20 mm., the latter being unusually large. R 15 mm. is nearer the normal maximum size of fissiparous individuals.

The species of *Sclerasterias* live in deep water, usually among rocks. All the specimens of fissiparous young which I have seen were taken by means of hempen tangles. The data for the following notes are admittedly incomplete. There is every reason to believe, however, that many years will pass before more material is forthcoming.

Of the thirty-six young of *S. euplecta* examined, nine, or one fourth, have five rays. None of these show signs of fissiparity although two have lost individual rays. All of the twenty-six fissiparous specimens have six rays. Of these six rays, three are usually smaller and represent the regenerating half.

In *S. heteropœs*, fourteen of the young have six rays and only one has five rays. Twelve out of these fourteen six-rayed specimens have four madreporites symmetrically placed, with two on either side of the plane of fission (the two opposite interradii through which the disk splits being without them). Thus each half, after fission, has 2 madreporites—one on either side of the central ray of the triad (Figs. 1–3). The exceptions are a tiny symmetrical specimen with R 7.5 mm., on which I can find only one madreporite; and a specimen with 2 pores on the regenerating half and only one on the original half (Fig. 3). *S. euplecta* has three or four madreporites in regenerating material where the new rays are large enough to have developed pores.

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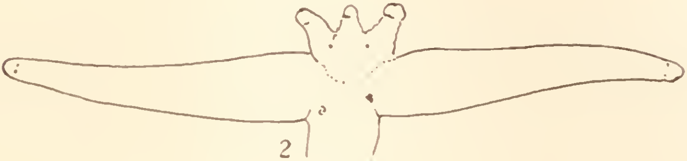
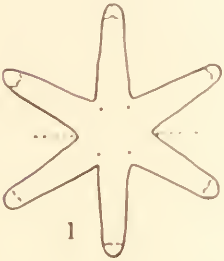
[4] It is not essential to introduce data to prove that these specimens are really young *Sclerasterias*, although there is ample evidence. In the fissiparous stage the crossed pedicellariae (which are like those of the adult) are not concentrated in circumspinal wreaths as in the adult, but are scattered between the spinelets. The latter are short, uniform, and two to four on each median radial plate and two or three on each superomarginal plate. In the adult only each alternate plate of the two series mentioned carries a single spine. The extra spines are absorbed.

[5] *R* is the distance between the center of disk and tip of ray—the major radius.





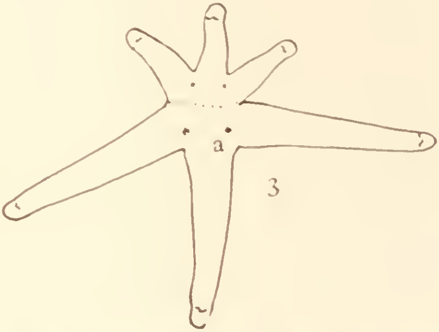


FIG. 1. *Sclerasterias euplecta.* The smallest symmetrical six-rayed specimen *R* 5.5 mm. The dotted lines indicate plane of cleavage. The four madreporites are indicated by dots; × 3.

FIG. 2. *Sclerasterias heteropæs.* Individual regenerating three new rays and two new madreporites; × 3.

FIG. 3. Same; a more advanced stage. In one specimen similar to this the madreporite *a* is lacking; × 3.

The only five-rayed specimen of *S. heteropæs* has two madreporites in nearly opposite interradii. Of the nine five-rayed examples of *S. euplecta*, eight have one madreporite and one has three madreporites (which are smaller than the single madreporite of the other five-rayed specimens).

It is clear that active fissiparity is correlated with six rays and with usually four symmetrically placed madreporites; for none of the five-rayed examples shows evidence of having split through the disk. In these only separate rays have been shed as in ordinary autotomy, the disk remaining entire with the five oral angles uninjured. In fissiparity two opposite oral angles are split neatly in twain.

The location of the madreporites with reference to the plane of splitting would provide two directly opposed "physiologically anterior" points (Cole) and would thus automatically favor an equal splitting of the disk. Crozier (20) regards the multiplication of madreporites at separated points on the disk of *Coscinasterias tenuispina* as furnishing an assurance that portions of the body separated by autotomy will each be provided with a madreporic canal. This seems reasonable. However, a large, non-fissiparous species, *Acanthaster planci*, with upward of sixteen rays has four to eight madreporites.

The utility of several madreporites in fissiparous species would appear to be clear. But as to origin, it is not evident in *Sclerasterias* that the extra madreporites are solely post-larval developments as a preparation for fission. Furthermore we have a transitory post-larval hexamerous symmetry to account for in a characteristically pentamerous genus. The six-rayed young with four madreporites may have descended from larvæ with four hydropores. If so it is likely that we have in nature the sort of hydropore duplication reported by Newman in laboratory cultures of *Patiria miniata* (Newman 21, 21*a*). This physiological twinning in the larva may be here a normal precursor to a subsequent post-larval "untwinning," by which the six-rayed, four-pored, fissiparous young becomes a five-rayed, non-fissiparous adult with one madreporite. [I have specimens showing this last stage, *before* the spines and pedicellariæ have assumed the fully adult reduction and concentration.] In other words some of the incentive to splitting may be due to a sort of physiological duality locked up in the young with six rays. The five-rayed young with one madreporite would naturally be derived from larvæ with one hydropore. Possibly the five-rayed young with two and three madreporites are descended from incompletely twinned larvæ; or again, they may already have accomplished the reduction division without showing outward signs.

Although the mechanics behind this curious condition are as yet material for speculation, the phenomenon itself seems to produce a fairly definite asexual generation following close on the heels of the larval stage. This asexual mode of reproduction is associated with an abnormal symmetry for the genus.

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THE AXIAL GRADIENTS IN HYDROZOA.

VII. Modification of Development through Differential Susceptibility.

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In the preceding paper of this series the physiological analysis of embryonic development in certain species of hydrozoa was begun with a study of the physiological gradients and their changes during the usual course of development (Child, '25). The present paper is a continuation of this analysis and is concerned with the modification of development resulting from the differential susceptibility to various agents of different levels of the polar gradient or gradients.

Material and Method.

Developmental stages of the leptomedusa, *Phialidium gregarium*, abundant in Puget Sound, constituted the chief experimental material. Since the medusæ shed eggs or sperm more or less continuously early developmental stages are readily obtained by keeping the sexually mature medusæ in large containers for a time and then collecting the embryonic stages from the bottom. Developmental material obtained in this way shows a certain range of stages, depending on the length of time the medusæ are kept in the container, but by using large numbers of medusæ sufficient material for one or more experimental series can usually be obtained within a few hours. With this procedure the stages range from newly fertilized eggs to more or less advanced cleavage or early blastulæ and still greater uniformity can of course be obtained by sorting the stages, but this is unnecessary for most purposes. A few experiments were performed with the early planulæ of *Gonothyræa clarkii* after their emergence from the gonophores.

The experimental procedure consisted in the exposure of the developmental stages to concentrations of agents which had been found by preliminary experiment to inhibit development to a greater or less degree but not to be directly lethal. Agents and indicated concentrations used are as follows: KCN, $m/20000$, $m/25000$, $m/50000$; HCl, pH 6.8 −, pH 7.2, pH 7.3, pH 7.4 +;[1] LiCl, $m/25$, $m/50$, $m/80$, $m/160$; ethyl urethane, $m/25$ and very dilute neutral red. All solutions were made up in sea water and the concentrations given are those indicated by amount of agent and volume of water used. For experiment finger bowls holding some 400 cc. were used. These could be brought under a binocular or even under low power of a compound microscope without removal or disturbance of the developing material. When volatile agents were used bowls with an edge giving good contact with a glass plate were selected, being completely filled with the solution and covered with the plate so as to exclude air. Atmospheric humidity being high, there was practically no loss of water under these conditions and certainly no appreciable loss of the agent since renewal was usually made daily.

The variations in experimental procedure may be grouped as follows: (*a*) continuous exposure to a certain concentration beginning with the earliest stages obtainable; (*b*) temporary exposure to a certain concentration beginning with the earliest stages, followed by lower concentration or by return to sea water; (*c*) early development in sea water, exposure to agent beginning at later stage (blastula, early planula).

The developmental stages of *Phialidium* are so susceptible to change in condition that some degree of developmental modification frequently occurs in sea water in the laboratory if conditions are not maintained near the optimum. For example, if the developmental stages remain too long in the container with large numbers of medusæ, differential inhibition of development results, the modifications bring similar to those induced by other means. Under these conditions there is a decrease in pH, resulting chiefly from the CO_2 production of the medusæ. There may also be some accumulation of other metabolic products and some decrease in oxygen content. When development takes place in standing water several centimeters deep without renewal some degree of differential inhibition and modification usually occurs at the bottom while attachment and development of hydranth-stem axes proceed in the normal manner (Child, '25) on the sides of the container near the surface. Here also there is some decrease in pH at the bottom. When development takes place in a layer of water only a few millimeters deep no such differences appear and development is wholly or almost wholly normal if the material is otherwise in good condition. Occasional cases of modification usually occur even in the best material kept as nearly as possible under optimum laboratory conditions. Such modifications doubtless result from factors affecting particular eggs or spermatozoa before development or from poor condition of certain individuals.

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[1] At the time these experiments were performed it was believed that the increase in H-ion concentration was the chief inhibiting factor in the acid experiments, but in the light of the recent work of Smith and Clowes ('24) it appears probable that the increase in CO_2 resulting from the addition of HCl to sea water is more important than the increase in H-ion concentration. In my experiments no attempt was made to determine CO_2 tension after addition of HCl.

Experiments with the medusæ as the inhibiting agents were performed by placing a number of the medusæ in small aquaria or bowls with the developmental stages and determining the pH colorimetrically from time to time.[1] The modifications produced by crowding, standing water etc., are exactly similar in character to those produced by the various other agents used. Some forty years ago Metschnikoff ('86) described as variations similar modifications in the development of *Mitrocoma*.

As will appear below, with the various agents and conditions used, developmental modifications result from the differential susceptibility of different body levels. All agents and conditions used inhibit development, the higher levels of the gradient being most inhibited. In the lower concentrations differential acclimation, or after return to sea water differential recovery may occur, the higher levels of the gradients acclimating or recovering more rapidly or more completely than the lower levels, as indicated by increased developmental activity.

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[1] The work of Smith and Clowes ('24) makes it probable that in these experiments, as in those with HCl (see footnote, p. 177) the chief inhibiting factor is the CO_2 resulting from the respiration of the medusæ, rather than the increase in H-ion concentration brought about by the dissociation of carbonic acid formed from a part of the CO_2. The fact that the decrease in pH is rapidly reversed on shaking the water with air indicates that CO_2 production is responsible for it, but it is possible that other products of medusa metabolism may play some part in the inhibition of development.

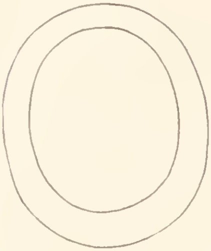
DIFFERENTIAL INHIBITION IN THE DEVELOPMENT OF THE PLANULA.

It is important to note first of all that there is no evidence of any specific effect on development of any agent used. The relations of the differential modifications to the gradient are similar for all agents, though the degree of differential action differs for different concentrations and for different agents, being greatest for LiCl, *i.e.*, in LiCl the high end of a gradient is relatively more, the low end relatively less inhibited than with other agents used. The high degree of differential action of lithium salts has also been noted by MacArthur ('24) in the modification of echinoderm development.

Since the modifications produced by the different agents are similar, detailed statements of the results obtained with each agent would involve much repetition and are omitted for the sake of brevity. The various degrees of modification are briefly described and data concerning their occurrence with different concentrations and agents are given.

The Lesser Degrees of Differential Inhibition.—In concentrations somewhat above those used for developmental modification the cleavage stages often separate into single blastomeres or collapse into irregular cell masses which sooner or later die, but in the concentrations used for modification of later stages, cleavage proceeds without appreciable alteration. Susceptibility is found to increase during early development and the lower susceptibility of cleavage stages as compared with later stages, together with the fact that more or less time is necessary for the inhibiting action to become evident, undoubtedly account for the absence of modification of cleavage with the concentrations used.

Figs. 1, 2 and 3 are diagrammatic outlines of blastula, immigration stage, and early planula as they appear under good conditions in the laboratory, and represent approximately "normal" development. Figs. 4–9 show the less extreme degrees of differential inhibition. These modifications are in general more frequent in material exposed to the lower concentrations from the beginning of development or to the higher concentrations from advanced cleavage or early blastula. They consist in different degrees of increase and extension of the basal thickening of the blastula wall and of all degrees of reduction, even to complete obliteration, of the blastocœl by continued mass ingrowth of the thicker parts of the wall instead of, or in addition to immigration



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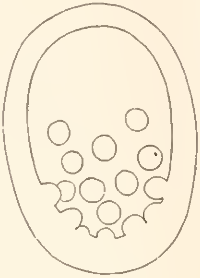

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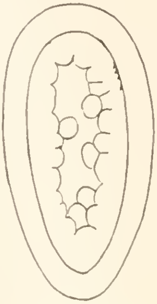

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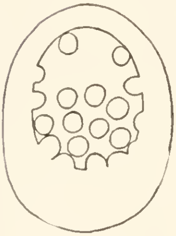

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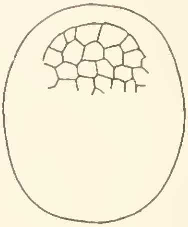

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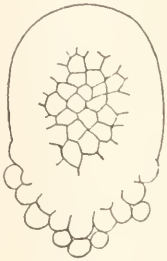

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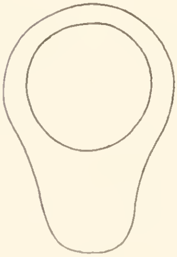

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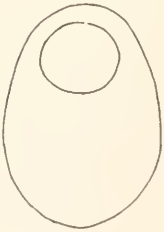

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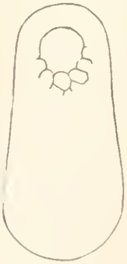

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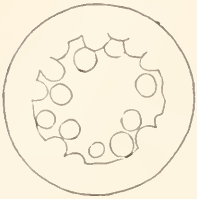
of single cells. The thickened region is a solid cell mass, usually without distinct boundary between ectoderm and entoderm. Fig. 6 represents a condition observed thus far chiefly in LiCl in which the blastocœl is completely obliterated by cells and basal emigration is occurring.

In all these modified forms development is retarded and locomotion is much slower than normal. Many of these inhibited forms advance slowly in contact with the bottom, being unable to support themselves free in the water, others revolve with little or no advance and some show reversal in the direction of locomotion (Figs. 6, 8). In these reversal of the susceptibility gradient and the reduction gradient (Child, '25) has also been observed.

These modifications represent an extension of basal characteristics and behavior toward the apical end, *i.e.*, a change of the more apical levels to the condition and behavior of the basal levels. The least degree of such modification consists merely in an increase of the basal thickening, an extension of the region of immigration toward the apical end and an increase in number of immigrating cells (Fig. 4). All gradations occur between this condition and forms in which almost the whole of the blastula wall is involved in immigration and ingrowth (Figs. 8, 9) and in some cases the cells of the basal region become so altered that they emigrate instead of immigrating (Fig. 6).

The differentially inhibited planulæ which result from earlier stages like Figures 4–9 are more or less elongated forms with excess of entoderm, often completely solid. Since they are usually not sufficiently active to swim free, locomotion takes place in contact with the bottom, in most cases with apical end in advance, but in some reversed, as noted above. As ciliary activity decreases these larvæ do not attach by the original apical end, but simply come to rest gradually with some meridian of the longitudinal surface in contact and more or less flattening occurs along this meridian. In the absence of differential acclimation or recovery development may go no further, or one or more stolon axes may develop slowly. In any case the body becomes enclosed in a delicate perisarcal secretion.

The More Extreme Degrees of Differential Inhibition.—These appear more frequently in the higher concentrations or in material subjected to inhibiting action from the beginning of development. They constitute a continuous series with the forms already described and are separated only for convenience. In material exposed to sufficiently high concentrations or degrees of action from the early cleavage stages, elongation of the blastula does not occur, the whole wall remains thick and immigration and mass ingrowth occur in all regions, resulting in a spherical solid larva, usually without distinct boundary between ectoderm and entoderm. Figure 10 shows an early stage, Figs. 11 and 12 show later stages of these forms.



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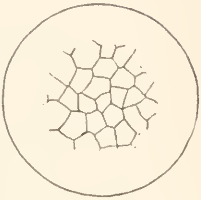

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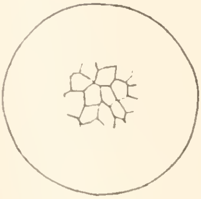

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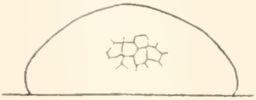

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When exposure to inhibiting agents is begun only after the blastula stage is attained, polar elongation, basal thickening and immigration may occur in the agent. In the higher concentrations, however, immigration or thickening of the blastula wall gradually extends apically and may involve all regions of the wall, the blastocœl may be obliterated and the elongation may disappear completely, the extreme modification short of death being spherical solid, apparently completely apolar forms indistinguishable from those described above (Figs. 11, 12). In these cases there is actual regression so far as visible axial differences, elongation and basal thickening are concerned.

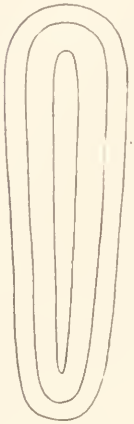
These spherical solid larvæ, whether they arise from earlier stages before elongation occurs, or from later elongated stages by regressive changes, are, so far as can be determined, apolar. Susceptibility gradient and reduction gradient are no longer present. They have lost the capacity for definitely directed locomotion and roll about on the bottom, being usually unable to support themselves free in the water. They show no definite attachment reaction, but gradually become quiescent and after movement ceases adhere by some part of the surface and become more or less flattened (Fig. 13). If conditions do not permit acclimation or recovery, no further development occurs, though they may live for two weeks or more. In short, the physiological differences constituting polarity have apparently been obliterated and the pattern is no longer axiate, but merely surface-interior (Child, '24, pp. 57, 93).

DIFFERENTIAL ACCLIMATION AND DIFFERENTIAL RECOVERY. Under "normal" conditions the blastula develops into the elongated free-swimming planula (Fig. 14), which shows at first an apico-basal gradient, but later develops at the basal end a second gradient opposite in direction to the first, as described in the preceding paper (Child, '25). Later the planula attaches itself by the original apical end and the hydranth-stem axis develops from the original basal end, *i.e.*, from the secondary gradient (Fig. 15). As pointed out in the preceding paper, the hydranth-stem axis represents a new polarity arising by a process of budding from the original basal end of the planula.

In the lower concentrations of agents which produce at first some degree of differential inhibition without completely obliterating polarity, differential acclimation or acquirement of tolerance may gradually occur in the course of a few days with further modification of development. Similarly differential recovery may occur on return to well aërated sea water after temporary exposure to concentrations which produce differential inhibition. Within a certain range of differential inhibition the regions most inhibited acclimate or recover most rapidly or most completely, as is indicated by further development. Since differential acclimation and differential recovery are secondary changes which take place following a differential inhibiting action, it is evident that they can modify only the later stages of development.

The first indication of differential acclimation or differential recovery in differentially inhibited planulæ which still retain some degree of polarity is the outgrowth of a stolon from one or both ends. Fig. 16, apical stolon, and Fig. 17, apical and basal stolons, as seen from above, show characteristic forms. In the case of Fig. 18 the larva probably came to rest with the body bent upon itself and gave rise to a stolon from each end, though it is possible that one or both of these stolons represent new polarities. In these and later figures the heavy line indicates perisarc, the light line the outer surface of the cœnosarc.

As I have pointed out in an earlier paper (Child, '23), hydroid stolons represent somewhat inhibited physiological gradients or reduced polarities and hydranth-stem axes can be easily transformed into stolons by inhibiting factors. In these planulæ stolons instead of hydranth-stem axes develop because in the earlier stages of acclimation or recovery a considerable degree of inhibition is still present. The apical region of the stolon axis is sufficiently active in relation to other levels to grow at their expense, but for the development of a hydranth-stem axis a higher metabolic rate is evidently necessary than for a stolon axis.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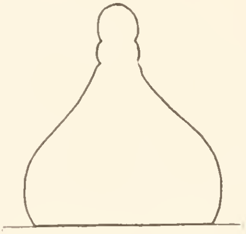
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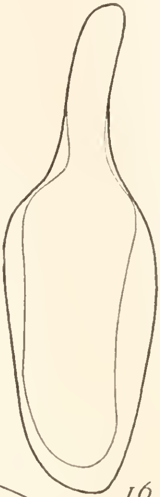
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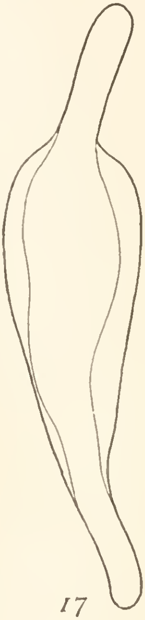
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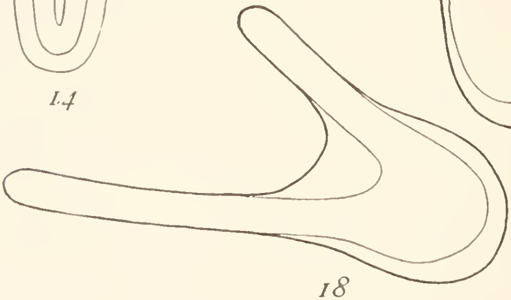
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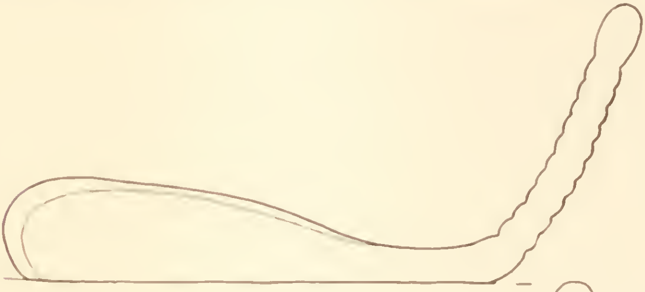
Figure 16 represents a larva in which the second gradient at the basal end did not develop before inhibition occurred, or was obliterated by the inhibition, while the original gradient was not wholly obliterated. In Fig. 17 and probably in Fig. 18 both gradients have persisted through the inhibiting action and both develop independently as stolons.

The further history of these stolon outgrowths depends on the degree of acclimation or recovery. When a certain degree of inhibition persists, *e.g.*, very commonly in acclimation, in which the material remains exposed to the agent, the stolon tips continue to grow at the expense of the more basal levels, which are gradually resorbed. Such growth may continue for weeks and extend over many centimeters, being ended only by exhaustion of nutritive material. It is discussed more fully in a later section.

When the degree of acclimation or recovery is such that the gradients attain or approach their normal physiological condition, hydranth-stem structures develop. In differential acclimation and recovery this condition is usually attained only gradually, consequently the first outgrowths are almost invariably stolons and these transform later into hydranth-stem axes as in Fig. 19, or give rise by budding to such axes as in Figs. 20 and 21. In these figures the animals are viewed from the side, the original larval body and the stolons are in contact with the bottom and the hydranth-stem axes are more or less erect.



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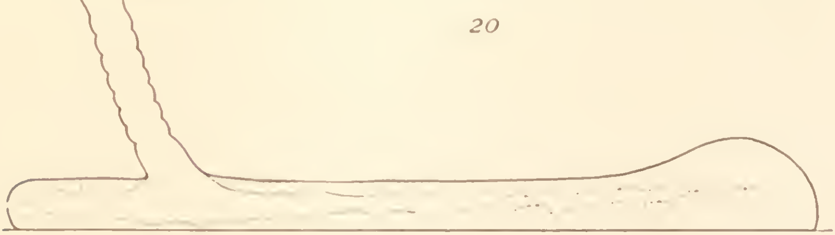

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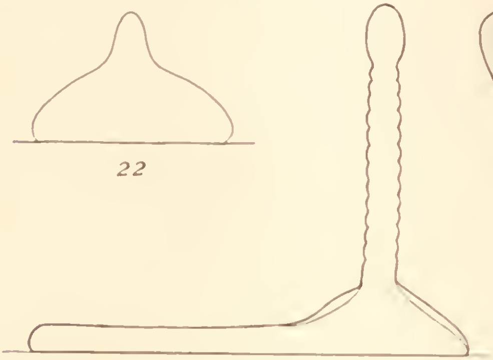
With the appearance of hydranth-stem axes the further growth of the stolon is usually inhibited or retarded until the hydranth-stem axis attains sufficient length to permit some degree of physiological isolation of the stolon tip. Often the stolon serves as nutritive material for the developing hydranth and stem and may be completely resorbed. Fig. 19 shows a case in which such resorption is going on. The stolon, originally filling the perisarc, is much reduced and irregular in outline, while in the hydranth-stem axis the cœnosarc fills the perisarc. Moreover, the stolon cœnosarc, particularly in the most reduced regions, is almost transparent, while that of the hydranth-stem axis appears more granular and denser. By means of inhibiting conditions these hydranth-stem axes may be again transformed into stolon axes or inhibited to such an extent that they serve as material for the growth of stolon axes and are resorbed. With the approach of exhaustion also, the hydranth-stem axes usually undergo resorption, while the stolon axes persist and grow, at least for some days longer.

The Appearance of New Polarities.

As noted above, the spherical, apparently completely apolar forms undergo no further development unless some degree of acclimation or recovery occurs and then they apparently develop new polarities. Occasionally in cases of rapid recovery a hydranth-stem axis arises directly from the upper free surface of the flattened mass (Fig. 22). In the absence of landmarks the possibility cannot be absolutely excluded that this outgrowth represents the hydranth-stem axis of normal development. On the other hand, observation indicates that the spherical forms may come to rest with any part of their surface in contact and that their polarity has been completely obliterated. If this is true, the hydranth-stem axis developing from the upper surface represents a new polarity determined by differential exposure of free surface and surface in contact, probably involving differences in intake of oxygen or the giving off of CO_2 or both.

Figure 23 shows in side view a case of simultaneous development of two axes from a spherical apolar form. The axis arising from the upper surface is a hydranth-stem axis, that from the side, a stolon axis. Since these axes develop from a spherical, apparently apolar form, it seems probable that both represent new polarities, the hydranth-stem axis being determined as a gradient by the greater respiratory activity occurring on the upper free surface, the stolon axis by some local region of greater activity on the margin.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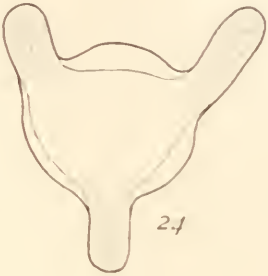
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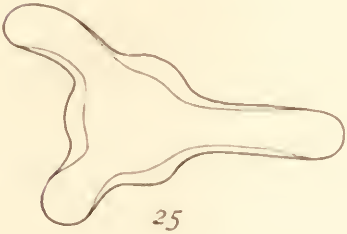
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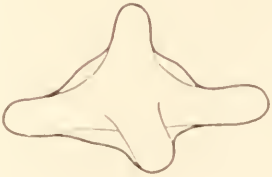
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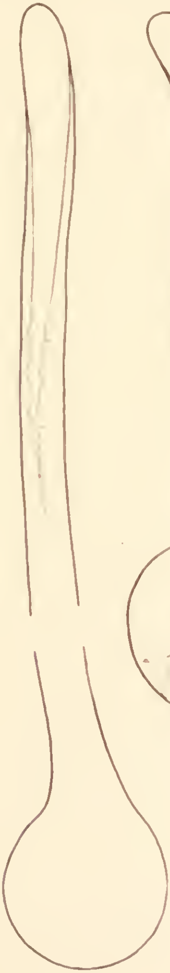
Usually, however, acclimation or recovery occurs so slowly that the outgrowths which arise from these spherical forms are at first stolons and only later transform into, or give rise to hydranth-stem axes. In many of these cases there can be no doubt that new gradients or axes originate since three and not infrequently four stolons may arise from a single individual (Figs. 24–26, all viewed from above). These axes, or at least some of them, are evidently "adventitious," *i.e.*, they are determined by slight local chance differences among the cells, each stolon originating as a localized region in which the cellular activity is greater than in surrounding regions. This region begins to grow at the expense of other parts about it and as it grows the gradation in activity, which is at first about a center becomes an axial gradient as growth proceeds. Each outgrowing stolon is such a gradient with high end at the tip. In Fig. 24 the basipetal gradient of cytolysis in lethal concentrations is indicated in the three stolons.

When acclimation or recovery proceeds far enough, the tips of such stolons sooner or later transform into hydranth-stem axes, like the stolon of Fig. 19, or hydranth-stem axes arise as adventitious buds from their upper surfaces, as in Figs. 20 and 21. In this way an individual which has been made apolar through differential inhibition may develop during acclimation or recovery several new stolon axes and each of these may later give rise to several hydranth-stem axes. On the other hand, if acclimation or recovery is only partial each stolon may continue to grow indefinitely as a stolon until exhaustion occurs.

THE GROWTH AND SEPARATION OF STOLONS.

The continued growth of stolons under slightly inhibiting conditions shows certain features of interest, some of which were briefly described in an earlier paper (Child, '23) as observed in stolons arising from hydroid colonies in consequence of inhibiting conditions. The stolons arising from the inhibited developmental stages grow at the expense of other parts and in time the whole substance of the inhibited larva may be used in the growth of the stolon or stolons arising from it, leaving only the empty perisarc in the position of the larval body and an empty perisarcal tube connecting this with the stolon. Fig. 27 shows a case in which an inhibited form gave rise to a single stolon, which finally used up the whole larval body in its growth. At the stage shown the tip of the stolon had reached a point several centimeters distant from its point of origin, only a small part of the perisarcal tube connecting stolon and larval body being shown in the figure. After using the substance of the larval body, the stolon tip continues to grow at the expense of its more basal levels. The cells of the tip remain in good condition, while those of the basal end become shrunken and transparent and undergo atrophy. In this way the cœnosarc of the stolon becomes shorter and shorter until only a small mass of tissue remains.

Figure 29 shows a case in which an inhibited form gave rise to two stolons near together. At the stage shown they have used the whole larval body and then their own substance to such an extent that they are entirely separate from each other, except for the perisarcal tubes, and each has branched. In the later history of this case the cœnosarc of the larger lateral branch and that of the main stolon from which it arose became separated and each pursued its own course, the tip continuing to grow at the expense of more basal regions. The shorter lateral branch on the other main stolon is undergoing resorption at the stage figured and later was completely resorbed. Occasionally a growing stolon leaves a part of the larval body behind as in Fig. 28. This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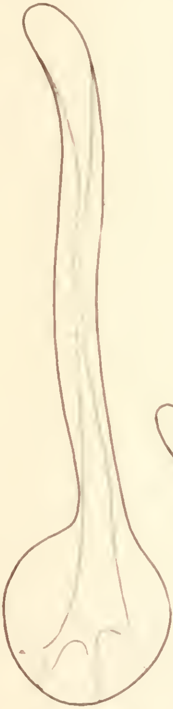
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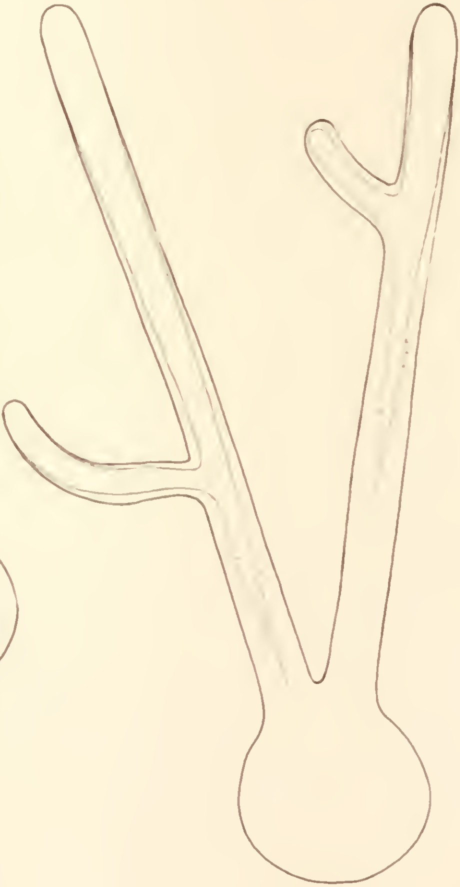
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apparently occurs when the stolon is not very active and the gradient is therefore short. Under such conditions the tip is apparently able to obtain nutrition only from regions within a short distance. The region in which atrophy occurs evidently represents approximately the limit of this distance.

In the earlier stages of this process of growth during starvation the development of hydranth-stem axes from such stolons may be induced by providing optimal conditions, but in later stages this transformation occurs less frequently and with the approach of exhaustion, but before stolon growth ceases, all attempts to induce transformation have thus far failed.

This continued growth of the stolon tip at the expense of the larval body and later of its own substance is an interesting case of a more active region maintaining itself at the expense of less active regions. Whether the atrophy of the less active regions represents primarily a more rapid autolysis or some other process, it is clearly evident that the most active region, the high end of the stolon gradient, succeeds in using the substance of all other levels, not only for maintenance, but for growth. Such stolons may grow over a distance of 10–15 centimeters before exhaustion occurs. Doubtless each stolon level lives at the expense of levels below it and sooner or later becomes a source of nutrition to levels above it in the gradient. This relation may mean simply that less active regions undergo autolysis more rapidly and that the more active regions are able because of their activity to use the products of autolysis in maintenance and even in growth.

A similar relation between different levels of the axial gradient appears in other cases of reduction by starvation. For example, in planarians and other forms undergoing reduction the proportions approach those of younger animals because the higher levels of the polar gradient, particularly the head region, maintain themselves to some extent at the expense of lower levels and so undergo reduction less rapidly than those.

Experimental Records.

The uniformity of result makes it entirely unnecessary to give the records of all experiments in full. A few characteristic records are given in Table I. as examples. Other records differ from these only as these differ from each other, *i.e.*, in degree and rate of differential inhibition, acclimation or recovery, according to the conditions of the experiment. The various forms, normal and modified, are indicated in Table I. by abbreviations as follows:

B. Normal blastula (Figs. 1, 2).
IB. Differentially inhibited blastula (Figs. 4–9).
Ap. Spherical or flattened apolar form (Figs. 10–13).
EP. Early planula (Fig. 3).
LP. Late planula, elongated (Fig. 14).
IP. Differentially inhibited planula, shorter, thicker than normal, with excess of entoderm.
H. Hydranth-stem axis developing directly from basal end of planula attached by apical end; "normal" hydroid development (Fig. 15).
NH. Hydranth-stem axis developing directly from apolar form (Figs. 22, 23).
StH. Hydranth-stem axis developing indirectly by transformation of, or budding from a stolon axis (Figs. 19–21).
St. Stolon axis (Figs. 16–18, 24–29).
Dd. Dead.

Each series of Table I. consisted of one hundred or more eggs or early embryos. All different forms observed were recorded at each examination and many drawings were made. In the table the relative frequency of the different forms is roughly indicated by enclosing in parentheses the designations of those forms which were present only in relatively small numbers (approximately 20 per cent. or less). The designations not so enclosed represent the characteristic forms of the series at the time indicated. As indicated at various points in the table the solutions were sometimes diluted after inhibition had occured, particularly when the appearance of the material indicated that development was not likely to proceed farther in the concentration originally used. Such dilution made acclimation possible or hastened it. The series given are characteristic. Frequent repetitions during the same and different summers showed nothing essentially different from the data presented.

Taking Series 4 I. as an example, the Table shows that in KCN, *m*/25,000 the forms after one day were inhibited blastulæ and

TABLE I.

Series.	Controls	2	4 I	4 II	3 I	3 II	9	8 III	5
Agent	water	KCN	KCN	KCN	HCl	water 2 days HCl	LiCl	LiCl	medusæ 2 days
Concentration		m/20,000	m/25,000	m/50,000	pH 7.4+	pH 7.2	m/80	m/160	pH decreased to 7.5
Days after beginning of experiment									
1	B EP	(B) IB Ap	(B) IB Ap	EP IP	IP Ap		IB (Ap) diluted 3/4	IB Ap	IB
2						EP (LP)		IP Ap	(EP) IP (Ap) medusæ removed; water
3		IP Ap returned to water	(IP) Ap	LP IP (Ap)	St Ap		(IP) Ap	IP Ap (St)	
4	H(StH LP)	(IP) Ap Dd	(IP) Ap diluted 1/3		St(StH Ap) diluted 1/3	(LP) St Ap diluted 2/3			
5		(IP Ap) Dd				St (Ap)		(StH St) Ap Dd	IP Ap
6	H (StH)		(IP) Ap	H StH St	StH St				St (Ap)
8							StH St Ap		(StH) St
9			(StH) St Ap						
12			NH StH (St Ap)						

apolar forms with a few normal blastulæ; after four days chiefly apolar forms with a few inhibited planulæ; solution now diluted one third; after nine days chiefly outgrowing stolon axes and apolar forms with a few stolon axes giving rise to hydranths; after twelve days chiefly new hydranth-stem axes arising directly from previously apolar forms and hydranth-stem axes arising from stolon axes, with a few stolon axes bearing no hydranths and a few apolar forms still remaining.

Modification of Development in *Gonothyræa*.

A few experiments with HCl and KCN, exposure beginning with the early planula after emergence from the gonophore, were sufficient to show that the modifications resulting from differential inhibition and from differential acclimation and recovery are the same in *Gonothyræa* as in *Phialidium*. To decrease in pH the susceptibility of *Gonothyræa* is less than that of *Phialidium*. For example, HCl, pH 7.4 has very little inhibiting effect on *Gonothyræa*, the planulæ usually attaching in the normal manner and giving rise to hydranth-stem axes without stolon outgrowths. Series 3 I. of Table I. shows that this pH is strongly inhibitory for *Phialidium*. For obliteration of polarity in *Gonothyræa* a concentration of HCl giving pH 7–6.8 was found to be necessary and as acclimation occurred, stolon axes developed and even gave rise to hydranths. As might be expected from these results crowding with the medusæ had little or no effect on development within the range used for *Phialidium*.

Discussion.

The differentially inhibiting effect of the agents on the blastula and early planula stages is sufficiently evident. In the normal blastula the region of basal thickening and of immigration represents the low end of the apico-basal gradient (Child, '25) and the effect of the agents is to bring higher levels of the gradient into a condition in which they behave like the basal region. In the extreme cases (Figs. 10–12) in which polarity is apparently obliterated, the whole blastula wall, including even the apical pole, behaves like the basal region. Taking this behavior as a criterion, it appears to be true that the whole blastula is reduced to the physiological level of the basal region. Evidently such a change in condition is differential with respect to the axis: the apical region is most, the basal region least affected. When this reduction of all regions to the same physiological level is complete, the physiological gradient is obliterated and the fact that such forms show no indications of polarity until new gradients are determined in them is additional evidence for the conclusion that the gradient constitutes the polarity.

The fact that the basal region usually shows growth and thickening far in excess of the normal may seem at first glance to conflict with the interpretation of these modifications in terms of differential inhibition, but, as a matter of fact such overgrowth is a consequence of the differential inhibition. The basal region is less inhibited than the apical, consequently it is more active relatively to the apical region in the inhibited than in the normal forms. This relatively greater activity enables it to obtain a larger proportion of the available nutritive substance and so to grow to a larger size, though more slowly than in normal development. Up to a certain degree the excess and extension of basal thickening increases with the decrease in apical, as compared with basal activity. But with more extreme degrees of differential inhibition all levels become more and more alike and more basal as regards physiological condition and differences of growth and development disappear, as in the spherical apolar forms. The complete disappearance of these differences and the reduction of the larva to a spherical apolar condition are more likely to occur when the inhibiting factor has acted from the early stages of development and has obliterated the gradient before the differences in behavior of ectodermal and entodermal regions have been established.

As pointed out above, actual reversal of the physiological gradient occurs in some cases, the reversal being indicated by reversal of orientation in locomotion and reversed susceptibility and reduction gradients. In such cases the differential inhibiting action of the agent is such that the original apical region is reduced to a lower level of activity than the original basal region. Since such reversal occurs only after basal thickening has begun, it may result in a great increase of that thickening. The cases of emigration of cells from the basal region (Fig. 6) suggest that the polarity of these cells, *i.e.*, the differences between the exterior and the interior pole, have also undergone reversal, so that instead of immigrating into the blastocœl the cells emigrate.

It may be noted in passing that the less extreme degrees of differential inhibition (Figs. 5–9) show interesting resemblances to the exogastrulæ and related forms such as solid blastulæ of the echinoderms, which also result from differential inhibition (MacArthur, '24). In both the hydrozoan and echinoderm modifications the entoderm—and in echinoderms the mesenchyme—is greatly increased at the expense of ectoderm, because the lower levels of the gradient are normally less active and therefore less susceptible and less inhibited than the higher levels. Actual reversal of the susceptibility gradient may also occur in both hydrozoan and echinoderm. The cases of emigration of cells (Fig. 6) correspond most closely to the exogastrulæ.

Spek ('18) has maintained that gastrulation is due to difference in the degree of swelling of internal and external parts of the cells concerned and that lithium salts and some other agents determine exogastrulation by reversing these differences. Whether or not this interpretation be correct, the differential action of the agents shows clearly the same relation to the gradient in both hydrozoan and echinoderm.

That the inhibition, even though it does not obliterate polarity, interferes with the usual sequence of events in later stages is shown by the fact that when acclimation or recovery occurs, the outgrowth may be apical (Fig. 16) or both apical and basal (Fig. 17), that is, the stolon may arise from the primary gradient or from both primary and secondary gradient and probably also from the secondary alone (Fig. 19). These differences undoubtedly depend on differences in stage of development at time of exposure and on degree of inhibition. The development of both apical and basal stolons indicates that under the inhibiting conditions the two ends have become independent of each other.

The conditions determining the "adventitious" polarities in the acclimation and recovery of apolar forms (Figs. 24–26) are obviously accidental for no two individuals are alike as regards localization and time of appearance of the different axes. Evidently these axes originate as local areas of slightly greater activity in some region or regions of the mass. Some of them may represent persisting traces of the original polarity or polarities, but some of them are certainly, and all of them may be new axes. The fact is of interest that in such forms adventitious stolon-axes may arise from any part of the free surface, but the direct origin of a hydranth-stem axis has been observed only from the most exposed portion farthest from the surface in contact (Figs. 22, 23).

The fact that the stolon axes usually appear first in acclimation and recovery and only later transform into, or give rise by budding to hydranth-stem axes confirms the conclusion of an earlier paper (Child, '23). In that paper it was shown that apical regions of various hydroid colonies can be transformed into stolon axes by slight degrees of inhibition or depression and that the stolons can again give rise to hydranth-stem axes with acclimation to, or recovery from the action of the agent. Such apical transformations do not represent reversals of polarity, but rather simply a depression of the gradient to a lower physiological level. In Lund's experiments on *Obelia* with electric current (Lund, '21) the position of the piece with respect to the electrodes merely determines whether the gradient arising at a given end shall be more or less inhibited and develop as a stolon or whether it shall attain the higher physiological levels characteristic of the hydranth-stem axis. Since axes, either stolon or hydranth-stem, very commonly arise from both ends of pieces without action of the electric current, it is evident that the current is not necessary for the determination of these polarities, even though it does determine their character as stolon or hydranth-stem axes and may, when acting, assist in determining the gradient at one end or the other of the piece. In any case the effects of the current are in no way specific as regards determination of the two sorts of axes, for, as I have shown, essentially the same results can be obtained with various chemical and physical agents in both hydroid colonies and developmental stages. Apparently all that is necessary to transform a hydranth-stem gradient into a stolon-gradient is a slight degree of inhibition, and the reverse transformation is accomplished by environmental changes in the reverse direction. In Lund's experiments with *Obelia* the anode apparently acts to some extent as an accelerating, the cathode as an inhibiting agent.

Physiologically the stolon does not represent simply the basal end of the hydranth-stem axis, but is itself an axis, a gradient, as has been demonstrated in various ways. Like new hydranth-stem axes, it originates from a bud and the high end of its gradient constitutes a growing tip instead of a hydranth. At the stolon tip synthesis of protoplasm, growth and cell division apparently keep pace with each other as in the growing tip of a plant, so that differentiation does not occur, but when the tip is raised to a higher physiological level by change in external conditions it loses the capacity for unlimited growth and differentiates sooner or later into a hydranth.

The demonstration that the stolon is a somewhat inhibited axis or gradient throws light on many otherwise puzzling features of the behavior of isolated pieces of hydroids and hydroid colonies. The stolon gradient may be inhibited, either by external conditions or by its nearness to a dominant hydranth region (Child, '15, pp. 91–2). Consequently a stolon axis may arise at a cut surface which is near vigorous hydranth regions and it may also arise under conditions which prevent formation or maintenance of hydranths. Pieces in deep standing water may produce stolons, those in shallow, or frequently changed water hydranth-stem axes. Pieces may produce first stolons, then hydranth-stem axes or *vice versa*, with slight differences in conditions of culture.

Three conclusions based on the evidence presented in this and earlier papers of this series and on the work of Lund and others are important in this connection: first, the stolon as well as the hydranth-stem represents an axis, a polarity, a gradient; second, the stolon gradient represents primarily lower levels of physiological activity than the hydranth-stem gradient and reversible transformation in either direction is possible and does not necessarily involve reversal of polarity; third, hydranth-stem or stolon axis may be determined according to conditions by the quantitative differential or local action of environmental factors independently of their specific constitution. These conclusions afford a physiological basis for the interpretation of data at hand and for further investigation and control of polarity.

SUMMARY.

1. Differential inhibition of planula development can be brought about in the hydrozoan, *Phialidium gregarium*, by KCN, LiCl, ethyl urethane HCl (probably CO_2) and by the presence of the medusæ (CO_2). Resulting modifications range from slight increase and extension of basal thickening of blastula wall followed by development of slightly modified planulæ to spherical, solid, apparently apolar forms. In these immigration and mass ingrowth are increased and take place from all parts of the wall, and further development occurs only if a new polarity arises.

2. In differential acclimation in low concentrations of inhibiting agents and differential recovery after return to water further development of persisting axes may occur or new polarities may arise through differential exposure of the surfaces of apolar forms or through "adventitious" localization of regions of greater activity. In rapid acclimation or recovery such axes may develop directly as hydranth-stem axes, but usually they are at first still more or less inhibited and develop as stolons and their tips transform later into hydranth-stem axes, or such axes arise by budding from their free surfaces.

3. The stolon represents a polarity, a gradient, at a lower level of physiological activity than the hydranth-stem axis. Transformation of hydranth-stem into stolon results from inhibition, of stolon into hydranth-stem from acceleration; neither involves necessarily a reversal of polarity.

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DIRECT AND AFTER EFFECTS OF CHANGES IN MEDIUM DURING DIFFERENT PERIODS IN THE LIFE HISTORY OF *UROLEPTUS MOBILIS.*

I. EFFECTS OF BEEF EXTRACT.

LOUISE H. GREGORY.

In 1905 Woodruff[1] as a result of his experiments with salt stimulation stated, "the conclusion seems to be justified that a given stimulus produces different effects at different periods in the life cycle." For the past two years while working on the problem of the nutrition of *Uroleptus mobilis*, I have had the opportunity of testing the sensitiveness of the protoplasm of different ages and find that so far as the division rate is concerned, the nature of the response to beef treatment and in all probability to treatment with potassium phosphate and other salts can be predicted if the age of the protoplasm is known.

The material used in these experiments was taken originally from the stock of Professor Calkins at Columbia, and the same series numbers were kept. From time to time conjugations occurred in the stock cultures at Barnard and new series were started in which case the letter *B* has been added to the series number to distinguish it from the Columbia series of the same number but possibly not of the same ancestry. In the present paper the results are given of experiments with beef extract only. In a later paper, the experiments with potassium phosphate and other salts will be reported.

A. The Direct Effects of Beef Feeding.

Professor Calkins[2] has cultivated *Uroleptus mobilis* for the past six years on a hay-flour medium, made "by boiling 100 mgs. chopped hay with 130 mgs. flour in 100 cc. Great Bear Spring water for ten minutes and diluting this when 24 hours old with equal parts of spring water." Woodruff and Baitsell[3] found that a .025 per cent. solution of Liebig's beef extract was a favorable medium for certain infusoria, and after experimenting with solutions of varying strengths, this percentage has been used in all of the following experiments with beef extract. A .05 per cent. solution of beef extract made up with Great Bear Spring water and a 24-hour-old solution of flour water of the same strength as in the normal hay-flour medium of Calkins, were used in equal amounts. This resulting medium was a solution of the most favorable beef strength and exactly similar to the normal medium except that the beef solution had been substituted for the hay.

In conducting the experiments one individual was taken from each of the five lines of the control series and placed in the beef flour medium. Both series were kept under the same conditions of light and temperature. They were transfered to fresh medium at the same times and differed only in the hay or beef element of the medium. As in all similar experiments the rate of division was taken as the indication of the vitality of the protoplasm.

Discussion of Experiments.

(*a*) *Direct Response of Young Protoplasm to Beef Extract.*

Table I. shows the results of feeding very immature series with the beef-flour medium. Six young series of five lines each were tested for their reaction to the substitution of beef extract for the hay in normal medium and in every case there was a lowering of the vitality indicated by the slowing of the division rate. The amount of the depression varied from 1.8 to 9.6 divisions in ten days for each line of the series. Every young line responded in the same way indication that in this period which in the six cases extended from the 5th to the 40th generation, the protoplasm was in a condition in which the normal metabolic processes were easily and immediately upset and the vitality lowered.

Table I.

Effects of Beef Feeding during the Period of Immaturity.

Series No.	Age in Gen.	Av. No. Divisions per Line in 10-day Periods, Beef Series.	Av. No. Divisions per Line in 10-day Periods, Control Series.	Amt. of Depression in Division Rate per Line in 10 Days.
82....	17–24	11	21.6	9.6
97....	11–34	17.6	20.8	3.2
98 B..	17–40	20	24.8	4.8
99 B..	11–30	15.2	17.6	2.4
100 B..	11–25	13.2	15.0	1.8
101 B..	5–23	13.6	18.4	4.8

(*b*) *Direct Response of Older Protoplasm to Beef Extract.*

Table II. shows the results of feeding *Uroleptus* with the beef-flour medium when the protoplasm was older than in the experiments shown in Table I. Series 97, 98*B*, 99*B*, 100*B*, 101*B*, all of which had been depressed in the beef-flour medium when tested earlier in their life history, now are stimulated by the treatment. Series 97 during the 60–75th generations showed an increase in ten days of 6.8 divisions per line over the control series. The stimulating effect of the beef was seen again though to a less degree in the 167th generation and even in as late a period as the 225th generation, the beef series maintained a slightly higher rate of division than the control. Series 98*B* depressed in the 40th generation, was stimulated in the 75th and 122d generations and at both times, the division rate exceeded that of the control. This same increase it vitality was observed in varying degrees in Series 99*B*, 100*B*, and 101*B* each one having been previously depressed by the beef treatment. In addition to the above experiments, three other series were tested whose reaction to beef in their earlier history was not known. Series 72 was in its 50th generation when it was obtained from Columbia. A series was treated with beef for 40 consecutive days and throughout the entire period from the 50th to the 140th generation, the stimulating effects of the beef were apparent. Even in the 180th generation the division rate of the beef series was equal to that of the control. Series 81 during the 56th–84th generation, was treated with beef for 20 consecutive days. During the first ten days the division rate was slightly less than that of the control. In the second ten period, however the beef series showed a more rapid division rate than the control. Series 95 was treated for 30 consecutive days, from the 50th–102d generation and at no time was there any evidence of stimulation. When the series was again tested in the 160th, 190th and 210th generations, the beef series responded with a much higher division rate than the control. Thus all eight series in their later life history responded to beef treatment with an increase in the vitality as indicated by a quickened division rate. The age at which this increase appears varies as would be expected. Series 99, 100, 101, all divided more rapidly in the beef medium at an early age, one as young as

27 generations. Series 72, 97, 98 showed the stimulating effects of the beef about the 60th–75th generation. Series 81 and 95 failed to respond to the beef until a still later age and might be considered slow maturing series. Series 95 gave indications of a long period of immaturity, showing distinct depression in the beef as late as the 102d generation. Beef apparently is a depressant in the early generations and a stimulant in later periods of the life history.

TABLE II.

THE EFFECTS OF BEEF FEEDING DURING A LATER PERIOD IN THE LIFE HISTORY.

Series No.	Age in Gen.	Av. No. Divisions per Line in 10-day Periods, Beef Series.	Av. No. Divisions per Line in 10-day Periods, Control Series.	Amt. of Increase in Division Rate per Line in 10 Days, Beef Series.
72	58–79	19.8	18.4	1.4
"	79–100	20.6	19.2	1.4
"	100–125	17.0	14.0	3.0
" . .	125–140	16.8	9.2	5.6
" . .	180–200	13.6	13.6	0.0
81 . . .	56–68	16.0	16.4	−0.4
"	68–84	16.0	14.0	1.4
95	50–70	18.0	18.0	0.0
" . . .	70–84	14.0	16.0	−2.0
" . . .	84–102	12.0	18.0	−6.0
95	160–175	19.6	16.6	3.0
"	190–210	22.4	17.8	4.6
" . . .	210–225	20.8	15.7	5.1
97 . . .	60–75	21.6	14.8	6.8
"	167–185	16.2	15.2	1.0
"	235–254	11.4	11.2	.2
98 B. .	75–97	19.0	17.8	1.2
" . .	122–136	16.0	14.0	2.0
99 B. .	40–60	17.4	14.4	3.0
" . .	75–90	13.4	12.6	.8
" .	95–105	11.6	11.8	.2
100 B.	27–58	17.4	16.4	1.0
" .	90–105	13.0	11.6	1.4
101 B. .	39–64	14.2	11.0	3.2
" . .	80–102	14.0	13.8	.2

(c) *Direct Response of Old Protoplasm to Beef Extract.*

Table III. shows the results of feeding old series with the beef-flour medium. Eleven experiments were carried out on protoplasm varying in its age from the 210th to the 370th generation and in every case the result was one of depression and a lowering of the vitality. Series 61 taken from Columbia stock in the 250th generation was depressed to such an extent in the beef-flour medium that its division rate was cut from 16.4 divisions in ten days to 7.6 divisions in ten days and during the next ten day period the series died while the control was dividing normally. Series 69 was treated for 40 days when the protoplasm was from 175–335 generations old, and at no time did the division rate of the beef series equal that of the control, the average rate per line for each ten day period being 9.8 divisions while that of the control series was 14.5. divisions. Series 95 failed to show indications of age until the 230th generation when the beef series divided in ten days on an average of 3.2 divisions less than the control. When this same series was again placed in the beef-flour medium in the 310th generation, the division rate fell to 6.0 divisions per line less than that of the control. Series 97 was tested for its reaction to beef six times in its life history and after the initial depression in the period of youth, the vitality was not lowered until the 260th generation. From this age on, there was a definite slowing of the division rate for the beef, flour series. Series 61, 69, 77, 88 were all old series when taken from the Columbia stock so there was no opportunity to test them when younger. In their old age however, they gave the expected response to the beef-flour treatment.

A comparison of the results of these three sets of experiments indicates that the protoplasm of *Uroleptus* responds in a definite manner to treatment with beef according to its age. The very young individual apparently does not adjust its self to the new medium, its metabolic processes are retarded and its vitality lowered. At a later stage in its life history it is able at least to adjust itself to the change in medium and to maintain a vitality as great as that of the control. Then appears an age when it is not only able to adjust its self to new conditions but is stimulated by them and shows an immediate definite increase in its division rate. Finally with age there is again a lack of adjustment indicated by the lowering of vitality and slow division rate. If the series is very old the substitution of the beef is sufficient to cause the death of the race.

Table III.

Effects of Beef Feeding during the Period of Old Age

Series No.	Age in Gen.	Av. No. Divisions per Line in 10-day Periods, Beef Series.	Av. No. Divisions per Line in 10-day Periods, Control Series.	Amt. of Depression in Division Rate, Beef Series.
61.....	360–370	8.8	16.4	7.6
69	275–335	9.8	14.5	4.6
72.....	200–225	11.2	12.0	.8
".....	225–236	11.4	12.0	.6
77.....	210–230	13.8	16.2	2.4
88.....	255–270	7.6	8.8	1.2
95.....	230–243	10.6	13.8	3.2
".....	280–290	6.2	8.0	1.8
".....	305–310	0.8	6.8	6.0
97.....	260–270	5.4	7.2	1.8
".....	270–283	2.6	6.6	4.0

No definite time can be set for the appearance of maturity or the onset of old age. If a definite favorable response to the beef treatment is an indication of maturity, it may occur as early as the 30th or as late as the 102d generation. For the average series it would appear about the 60th generation which agrees with the statement of Calkins that the period of youth or immaturity covers about the first 60 generations. Old age usually shows its self about the 225th generation but it may appear as early as the 200th or as late as the 260th generation. Old age and immaturity outwardly are alike in their response to beef treatment. They also seem to agree in their relative duration. If the period of youth is prolonged as in series 95 the period of old age is likewise longer than that of the average. Series 95 lived 350 generations and showed indications of old age during the last 120 generations, Series 97 matured in about the average time of 60 generations and showed evidences of age only in the last 40 generations, the race dying in the 300th generation.

B. THE AFTER EFFECTS OF BEEF FEEDING ON PROTOPLASM OF DIFFERENT AGES.

In a number of experiments after a series had been kept in the beef-flour medium for ten days, one individual from each of the five lines of the series was put back into normal medium and the division rate of the new series compared with that of the series continued in the beef-flour medium.

Three young series after ten days in the beef medium were transfered to normal medium and in every case the vitality was increased to such an extent that it exceeded that of the beef series and equaled that of the normal control series. In other words the depression while in the beef medium was followed by a stimulation on the return to normal medium.

Four mature series were treated in the same manner some twice and others three times, and in one experiment only was there any indication of a stimulus when the series was transfered from the beef to normal medium. In five experiments the transfer was followed by a distinct lowering of the division rate and in three other experiments, there was no change in vitality as indicated by the division rate.

Finally series 95 and 97 were tested twice each in their old age and in all four experiments the transfer to normal medium was followed by a distinct increase in vitality.

Here again is seen the similarity in the behavior of immature and worn out protoplasm. In both cases there is an immediate depression of the life processes when treated with the beef, but this depression disapears on removal to normal conditions and in some cases there follows a period of higher vitality, the real effect of the beef being only delayed in its appearance. On the other hand the mature individuals respond immediately with a more rapid division rate which falls when the stimulus is removed and the medium is again normal. Immature protoplasm and protoplasm of old age are apparently not able to adjust themselves quickly to changes in the environment. They are at once depressed in the new condition but overcome the depression when back in normal medium and often show a quickened vitality. Mature protoplasm is like a healthy muscle that is always in tone, ready to respond to a stimulus and needs no time for adjustment to the changed conditions, thus the effects appear at once and would not be expected to continue when the cause has been removed. It may be concluded that beef is always more or less of a stimulant to Uroleptus but the time when the stimulating effects are apparent varies with the age. Mature races will respond at once. Immature and old series are at first depressed and show the stimulating effects only after they are once more in their normal environment.

TABLE IV.

AFTER EFFECTS OF BEEF FEEDING.

Series No.	Age While in Beef.	Av. No. Divisions per Line in 10-day Periods, Transfered Series.	Av. No. Divisions per Line in 10-day Periods, Beef Series.	Effect of Change from Beef-Flour to Normal Medium.
82 . .	17–24	18.0	5.4	Stimulation
99 B	11–30	16.8	16.2	"
101 B	24–34	15.2	13.8	"
98 B	75–97	15.6	16.0	Depression
"	122–136	12.6	16.0	"
" .	158–170	11.2	12.2	"
99 B .	40–60	15.6	15.6	No effect
" .	80–105	11.0	11.6	Depression (slight)
" .	105–120	10.8	10.2	Stimulation (slight)
100 B	37–58	14.2	14.2	No effect
"	90–105	10.6	13.0	Depression
101 B	39–64	14.0	14.0	No effect
95 .	280–290	8.2	7.8	Stimulation
" . .	305–310	5.0	0.8	"
97 . . .	235–255	8.0	7.4	"
" . . .	270–84	8.2	2.6	"

SUMMARY.

The response of *Uroleptus mobilis* to treatment with a beef-flour medium varies with the age of the protoplasm. Both young and old series are immediately depressed in the beef medium but recover their vitality and may show a greater division rate than that of the control series when transferred to the normal hay-flour medium. Mature series on the other hand are stimulated when placed in the beef medium. The stimulation however is not continued when the series is returned to the normal environment. Beef seems to be an immediate stimulant if the protoplasm is mature, and a delayed stimulant if the series is young or old, the effect being seen only after transference to normal hay-flour medium.

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Effects of Different Tensions of Carbon Dioxide on Certain Orthoptera (Grasshoppers).[1]

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Introduction.

Much attention has recently been given to the physiological effects of carbon dioxide upon mammals, while comparatively little information in this respect seems to exist for invertebrates. Because of this lack of data it seemed desirable to study the physiological significance of this gas for certain insects. The present paper is based upon a quantitative study of the resistance of the grasshopper to different tensions of carbon dioxide noting particularly the muscular movements displayed under the influence of the gas, the rate of recovery from its effects, and respiratory changes in an enclosed space, as measured by carbon dioxide and oxygen changes.

I wish to thank Doctor J. H. Bodine, under whose direction the work was done, for suggestion of the problem and for inspiring criticism throughout.

Material and Methods.

It has been shown in a recent paper by Doctor Bodine (1) that grasshoppers have a relatively high rate of carbon dioxide output and this fact suggested that they might be favorable material for determining some of the effects of carbon dioxide on insects. In carrying out the following experiments two species of *Melanoplus* were used: *Melanoplus differentialis* and *Melanoplus femur rubrum*.

Inasmuch as the methods employed are so varied, individual descriptions are given under each of the several experiments.

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[1] Data in this paper taken from a thesis submitted to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Master of Science.

In order to prevent confusion of connotation of the words used in describing the endpoints in the experiments the two following terms are defined:

1. First movement—designates the first reflex, *i.e.*, crawling or stirring of the animal after it has been removed from the influence of the gas.

2. Recovery—indicates that the animal has returned to a normal state.

EXPERIMENTS.

1. *Effects of Pure Carbon Dioxide.*

The animals used in these experiments, *Melanoplus differentialis* and *Melanoplus femur rubrum*, were raised entirely under laboratory conditions.

TABLE I.

SHOWING VARIATION IN TIME TO FIRST MOVEMENT AFTER SHORT EXPOSURES TO PURE CARBON DIOXIDE.

Animals:	*Melanoplus* (*differentialis* and *femur rubrum*) Adults									
Exposure Minutes:	1				2			5		
Species:	*M.diff.*	*M.f.r.*	*M.f.r.*	*M.f.r.*	*M.diff.*	*M.f.r.*	*M.f.r.*	*M.f.r.*	*M.f.r.*	*M.diff.*
Sex:	♀	♀	♀	♀	♀	♀	♂	♂	♂	♂
Number of Exposure.	Minutes to First Movement.									
1	7	4	4	5	4	4	2	6	11	13
2	4	5	4	7	3	2	2	8	10	5
3	5	4	5	8	2	2	2			
4	6	5	4	7						
5	5	5	Aver. 5.2		Aver. 2.6			Aver. 9		

Adults and nymphs of both sexes were placed separately in airtight tubes connected with a Kipp generator. The carbon dioxide was made by the action of hydrochloric acid on calcium carbonate, passed through concentrated sulphuric acid, pyrogallol and distilled water, thence into tubes containing the animals, and finally, into distilled water and out to the air. The animals were exposed to the steam of pure carbon dioxide for definite lengths of time ranging from one minute to twenty-four hours. They were then removed and the time to the first movement noted. The character of the movements from the beginning of the exposure until the animals had recovered was also observed.

Table I. shows the range, especially in shorter exposures, in minutes to the first movement. The variations shown are due perhaps to individual differences. From an examination of this table it may also be seen that when the animal is subjected to the influence of pure CO_2 for one minute and then removed, the time to the first movement varies from four to eight minutes with an average of 5.2. With two minutes exposure the time to the first movement is from two to four minutes with an average of 2.6. With five minutes exposure the variation is greater, being from five to thirteen minutes with an average of nine. Above five minutes exposure, that is from ten minutes to about three hours, there is less variation. With five hours exposure, there may be no response, and if any, the movements start immediately upon removal from the gas.

TABLE II.

SHOWING TIME AND RATE OF THE FIRST MOVEMENT AFTER EXPOSURE TO THE GAS FOR DEFINITE PERIODS OF TIME.

Exposure, Time in Minutes.	Time to First Movement; Average—Minutes.	Rate of First Movement, per Minute Exposure.
1	5.1	5.1
2	5.1	2.5
5	4.4	0.88
10	6.3	0.63
15	5.0	0.33
20	3.8	0.19
22	6.0	0.27
25	5.0	0.20
30	4.0	0.13
40	5.6	0.35
60	7.9	0.13
90	1.5	0.016
120	7.93	0.066
180	21.5	0.12
320	6.2	0.017
5 hours	Immediate	0

Table II. shows the average time in minutes to the first movement and the calculated rate per minute exposure to the gas. The data presented in this table give results for about two hundred animals exposed for varying periods. It is evident from an examination of this table and from Fig. 1, which shows the same results graphically, that the rate of first movement per minute exposure is apparently inversely proportional to the length of time the animals are exposed.

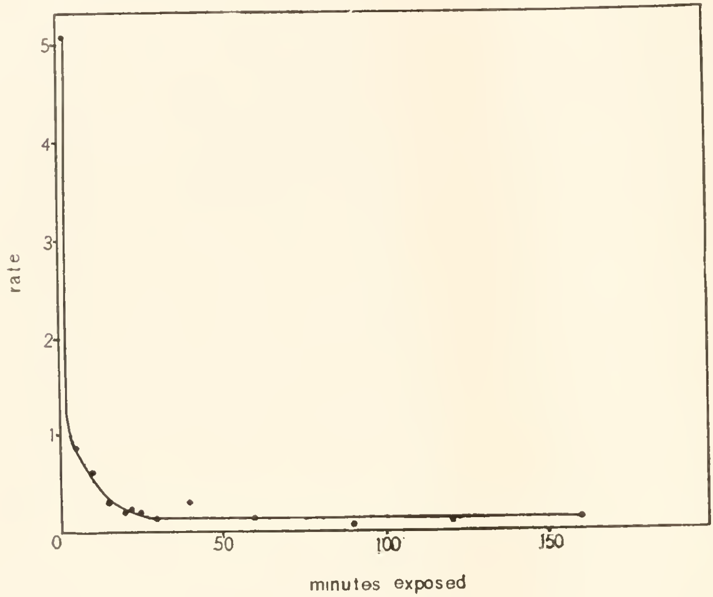

FIG. 1. Abscissa: The length of time in minutes the animal is kept under the influence of pure CO_2. Ordinate: Rate in minutes per minute exposure to the gas, of the first movement. (For further description see text.)

From Table II. and Fig. 1, it may also be seen that up to about thirty minutes exposure to the gas the change in rate of the first movement is rapid; above this there is no perceptible change.

It seems that the length of exposure to carbon dioxide, when above one hour, has an effect on the time to the first movement. Below this time limit, variations may be considered as due to individual differences. Perhaps a thousand determinations would be necessary to establish each point on a perfect curve. However, individual differences vary within limits and the data from the experiments performed are sufficient to determine the trend of the curve, as is given in Fig. 1.

In order to determine the relation between the first movement and recovery, animals of the same species, age and sex as above, were exposed to a stream of pure carbon dioxide for definite lengths of time ranging from one minute to five hours. The animals were then removed from the tubes, the time to the first movement as well as the time until the animals recovered was noted.

TABLE III.

SHOWING RELATION BETWEEN FIRST MOVEMENT AND RECOVERY AFTER EXPOSURE TO THE GAS FOR DEFINITE PERIODS.

Exposure, Minutes.	*A* First Movement, Minutes.	*A′* Rate of First Movement per Minute Exposure.	*B* Recovery, Minutes.	*B′* Rate of Recovery per Minute Exposure.
1	3.03	3.03	9.65	9.65
4	1.9	0.46	7.7	1.9
10	6.6	0.66	11.7	1.2
15	5.0	0.33	11.0	0.73
20	4.5	0.22	19.7	0.98
40	5.6	0.14	14.2	0.35
60	10.6	0.17	37.5	0.62
2 hours	13.27	0.11	39.0	0.32
3 hours	21.5	0.11	34.0	0.19
5 hours	—	—	—	—

Table III. gives the average values obtained from 45 animals, and 37 exposures to the gas and 136 endpoints, several animals being used in each exposure.

Generally, it may be said, from the data of Table III. and Fig. 2, curve *B*, that the longer the exposure, the longer the time required for the animal to recover. As for the time relation between the first movement and final recovery, there is sufficiently little variation as seen in Table III., columns *A* and *B*, to assume that within the limits of one to forty minutes exposure, the animals are affected, physically or chemically, in a similarly reversible manner. However, the longer exposures take a relatively longer time for recovery.

Columns *A′* and *B′* of Table III. and curves *A′* and *B′* of Fig. 2, show the rate of first movement and of recovery, per minute exposure, to follow the same type of curve. This perhaps signifies that reversibility is of the same nature in all cases. Variation in points determined, preventing regular curves are attributed to individual physiological differences in response.

Some idea of the striking characteristics of the movements of the grasshoppers produced during and after they are under the influence of carbon dioxide, may be derived from the following paragraph.

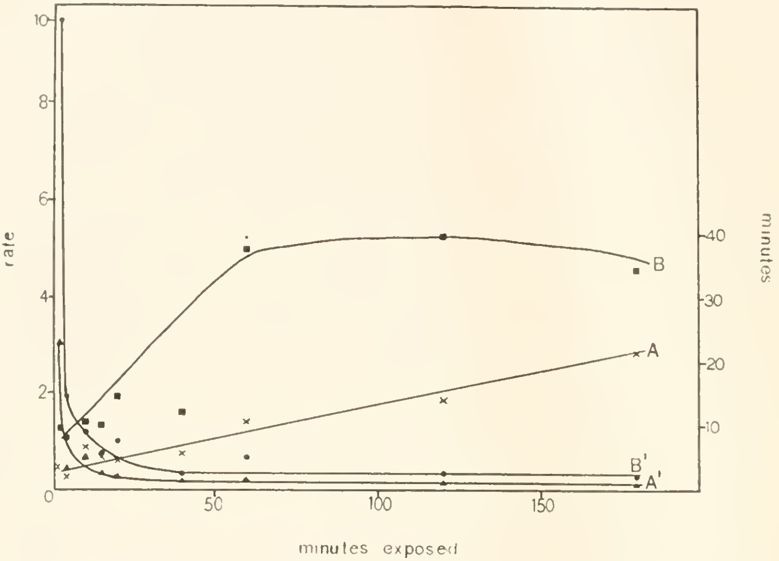

FIG. 2. Abscissa: Length in minutes of exposure to the gas. Ordinates at left, rate p. min. exposure. At right, time in minutes.

Curve *A* = Time in minutes to the first movement.
Curve *B* = Time in minutes to the recovery.
Curve *A′* = Rate of first movement per minute exposure.
Curve *B′* = Rate of the recovery per minute exposure.

(For further description see text.)

When an animal is subjected to the gas, it undergoes violent reflexes in all appendages—at times, the abdomen, due to stimulated respiratory movements, curls and uncurls; however, some animals make no movements. The period before asphyxiation occurs is less than one minute, usually about twenty to thirty seconds. After asphyxiation the animal becomes stiff or limp, depending upon the individual, and apparently lifeless, although some may exhibit slight reflexes. They remain in this condition until removed from the gas. In longer exposures some animals secrete an excessive amount of saliva and the antennæ droop. The salivary secretion may occur at any stage or upon removal from the gas. The first movements after removal from the gas vary with the individual. Usually there are slight reflexes, or quivers and tremblings of the legs, the large hind legs often being the first to move. After these reflexes have continued for a few seconds, becoming more and more pronounced, the legs become either stiff or limp. This latter condition may be followed shortly by a return of the reflexes and after longer exposure the reflexes may become violent. When the reflexes have subsided the animal begins to crawl feebly, slowly recovers its strength and appears normal. After shorter exposure the response differs, the first movement after removal from the gas may be a crawl or a hop, followed by normal movements.

Often it is impossible to compare the resistance of the animals when time alone is considered with regard to an arbitrary endpoint. However, if the first movement is taken as the result of a stimulus brought about by the interchange of gases when a new equilibrium is being established it may be a true endpoint for comparing resistances to different length exposures to carbon dioxide. As for a comparative resistance of the animals to carbon dioxide taking into consideration the character of the movements, as numerical data alone do not suffice, it may be said that: (1) *Melanoplus differentialis* is more resistant than *Melanoplus femur rubrum;* (2) nymphs of both species are more resistant than adults, they do not succumb so readily and recover more completely in a shorter length of time. Adults undergo the most violent reflexes, secrete more saliva and take the longest time to recover fully; (3) molting or freshly molted animals succumb most readily and recover very quickly from reversible doses, perhaps due to the softness of the chitin; (4) no sex differences were observed; (5) starvation for twenty-four hours has no appreciable influence on the recovery from the effects of the gas.

2. *A Measure of the Change in Percentage of Carbon Dioxide and Oxygen, Due to Respiration or Some Interchange of Gases between the Animals and the Air in an Enclosed Space.*

The following readings are based upon *Melanoplus differentialis*, nymphs and adults of both sexes being used.

A Haldane gas analysis apparatus was used for determining the per cent. of oxygen and carbon dioxide. The type of container used was as follows: A 160 cubic centimeter bottle, with a neck 1.5 centimeter in diameter was fitted with a rubber stopper carrying a 10 centimeter long capillary tube reaching within one centimeter of the bottom of the bottle. The outer end of the capillary tube was fitted with a piece of heavy rubber tubing carrying a clamp. This tube was fitted over the capillary of the Haldane apparatus when the air to be analyzed was taken into the burette of the apparatus.

In making a determination, an approximately known volume of carbon dioxide (made as described above) was transferred from a nitrometer tube to the container filled with atmospheric air and open to prevent increased pressure. The container was then closed and a known amount of air, about 8 cubic centimeters was withdrawn from it into the Haldane apparatus—previously filled with nitrogen made by removing oxygen and carbon dioxide from atmospheric air. The percentages of oxygen and carbon dioxide were then determined from this sample. The stopper was withdrawn, the animal introduced and the bottle quickly closed. Probable error at this point is negligible because of the slow diffusion of carbon dioxide. Since the air in the bottle was put under slight negative pressure in the first analysis, it was thought that some outside air might enter. However, immediate analysis showed no change great enough to affect the results of the experiments. Not more than three to five readings were made on each bottle so that negative pressure produced would not influence the normal reaction of the animal.

By the above method, using the Haldane apparatus, determinations are accurate to 0.02 per cent. Thus the results after taking into consideration individual differences, should be comparable to at least 0.1 per cent.

TABLE IV.

SHOWING PERCENTAGE CHANGES IN CO_2 AND O_2 DURING EXPOSURE TO THE GAS FOR DEFINITE PERIODS OF TIME.

Exposure.	Per Cent. Absorbed by Animal.		Per Cent. Given Off from Animal, O_2.	Per Cent. Difference Given Off from Animal, O_2.
	CO_2.	O_2.		
5–11	1.84	0.95	2.38	1.43
27–36	3.99	2.43	2.78	0.35
48–73	5.68	1.01	0.99	0.02
3 hours	10.03	1.53	0	—

It was found that with an original range of 28 to 32 per cent. carbon dioxide and 12 to 13 per cent. oxygen there was but a slight difference in the final composition of the air within the bottle. Table IV. is a compilation of data within this range. It is evident from this table and curve 1 of Fig. 3 that carbon dioxide is at first rapidly, then gradually, absorbed by the animal. An initial gain in oxygen as shown in the same table and curve 2 of Fig. 3 may be due to the slight negative pressure, some of the oxygen diffusing out of the tracheal tubes of the animal. However, later it is reabsorbed.

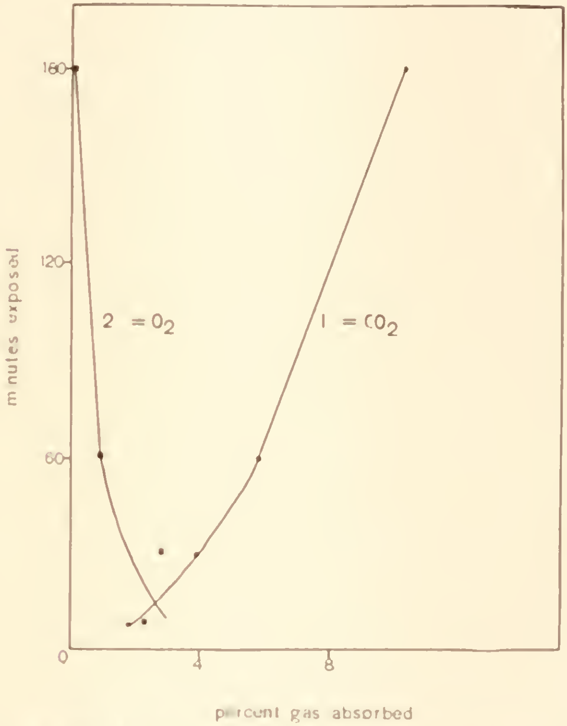

Fig. 3. Abscissa. Curve 1: Per cent. gain of CO_2 in tissues of the animal. Curve 2: Per cent. loss of O_2 from tissues of the animal. Ordinate: Length of exposure in minutes. (For further description see text.)

Table V. gives data for varying percentages of carbon dioxide combined with varying oxygen percentages. Column 3 of this table gives the ratio of carbon dioxide to oxygen in each case. In column 4 the lengths of time in minutes the animals were exposed to the gases are noted and in columns 5 and 6 the attendant

Table V.

Showing Changes in O_2 and Co_2 during Various Periods of Exposure to Varying Tensions of O_2 and CO_2.

Original Per Cent.		Ratio CO_2/O_2.	Exposure, Minutes.	Absorbed by Animal—CO_2.	Given Off by Animal—O_2.
CO_2.	O_2.				
7.95	18.32	0.44	6	0.64	−1.58
			27	0.43	0.51
			105	1.49	1.11
			143	1.44	0.92
29.82	13.30	2.24	10	3.63	1.1
			20	2.14	1.86
			73	4.71	1.60
29.39	12.73	2.31	11	0.35	0.54
			30	−0.42	1.29
			65	2.01	−0.69
			238	7.26	0.22
			252	11.98	−4.32
32.77	13.23	2.48	10	2.22	−1.81
			36	2.58	2.00
30.28	10.28	2.94	30	0.5	−2.00
			180	3.51	−2.05
			5.5 hrs.	2.57	3.71
35.94	11.75	3.07	10	1.31	−0.27
			49	8.89	−3.67
41.09	7.80	5.27	10	4.78	3.01
			27	5.09	−0.20
			48	8.22	−1.30
51.88	8.64	6.01	5	4.57	2.57
			29	7.88	4.78
			74	9.82	1.78

changes in carbon dioxide and oxygen percentages are given. Figs. 4 and 5 show these results graphically. Following the changes in per cent. of oxygen and carbon dioxide as shown in this table as well as the curves in Figs. 4 and 5, it appears that with a constant percentage of oxygen, provided it is within the limit of 7 to 18 per cent., the rate, per minute exposure, of carbon dioxide absorption increases with increasing carbon dioxide percentages. In addition, it may also be said that with 75 to 100 per cent. carbon dioxide no measurable interchange of gases takes place.

As previously stated, oxygen may at first be given out by the animal; however, in some cases it is absorbed. This absorption

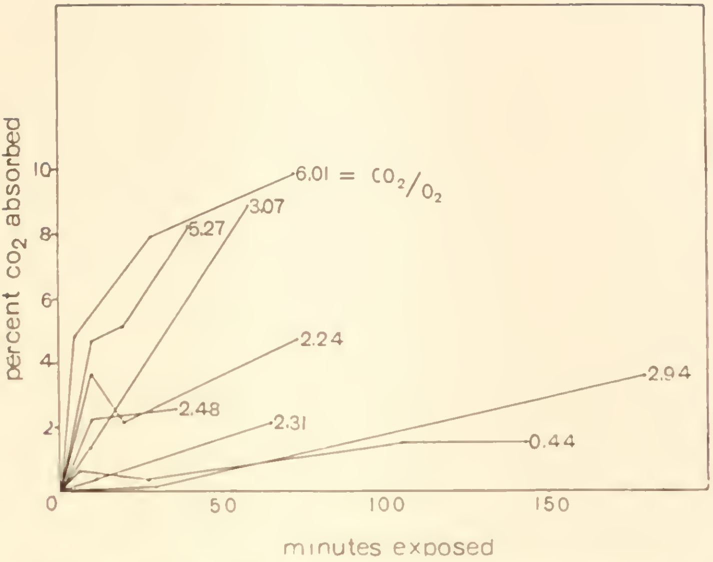

Fig. 4. Abscissa: Length of exposure in minutes. Ordinate: Per cent. CO_2 absorbed by the animal. (For further description see text.)

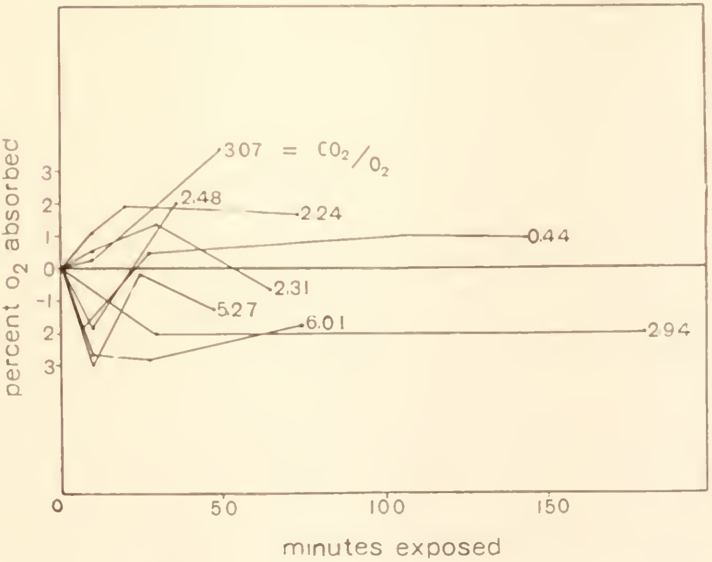

Fig. 5. Abscissa: Length of exposure in minutes. Ordinate: Per cent. O_2 absorbed by the animal. (For further description see text.)

or giving out of oxygen is found to occur within the first 30 minutes of exposure to the gases, but as the period of exposure increases, the gas is reabsorbed.

DISCUSSION.

Respiration in insects depends upon the supply of air in the system of tracheæ or tubes ramifying through the body. Equilibrium is maintained by muscular movements of the abdomen as well as by diffusion of gases and, for this purpose, eight separate segments of the abdomen, on either side in a ventro-lateral position, bear a spiracle or small tracheal opening through which the air in the tubes is periodically changed.

The relation between the respiratory and circulatory systems of insects differs from that of the vertebrates and is of such an arrangement that the blood seems to have little to do with the transportation of oxygen and carbon dioxide except in a purely mechanical way. In the grasshopper, the body fluid is kept circulating by means of a tubular heart lying along the dorsal side of the body cavity and as there are no blood vessels, the fluid directly bathes the tissues. Thus, it is by means of the minute tracheal vessels of the respiratory system that the gases are carried into the body and diffuse through the delicate tracheal membranes into the blood and tissue cells.

In a qualitative work on the influence of gases on the respiratory movements of grasshoppers, Walling (2) found that hydrogen inhibits the respiratory movements, then the animal partially revives and will continue to live in the hydrogen atmosphere for as long as four days. In oxygen the animal may be either active or dormant, depending upon the individual and, finally, dies from lack of food. Carbon monoxide reacts similarly to carbon dioxide and the animal will recover after longer exposures to the gas. As for carbon dioxide she found respiratory movements to cease within twenty to thirty seconds, but they returned when the animals were removed to fresh air. They could endure long exposures.

The characteristic behavior exhibited by the animals in the present experiments under the influence of carbon dioxide is perhaps due to physical changes in the tissue cells. It is known that carbon dioxide and oxygen may exist in solution in their body fluid. Consequently with an excess of carbon dioxide it is quite probable that much of it is absorbed by the tissue cells. Jacobs (3) has demonstrated the easy permeability of the cell membranes to carbon dioxide and in studying the effect of carbon dioxide on protoplasmic viscosity of *Paramecia* and of *Arbacia* eggs, he found that shorter exposures to the gas cause a great decrease in viscosity while longer ones cause an increase and within limits both are reversible (4). Hence changing viscosity of the protoplasm of the grasshopper muscle cell attended by liquefaction or gelation may produce the limpness or stiffness of the animal.

There may also be a chemical explanation of the effects of the gases on the animals. Carbon dioxide, by going into solution in the blood increasing the hydrogen ion concentration, or by acting in an undissociated state, may stimulate the nerve ganglia in the abdominal segments causing breathing movements, thus producing an effect similar to that caused by stimulation of the respiratory center in mammals as demonstrated by Hooker, Wilson and Connett (5) and by Scott (6).

Another theory is suggested by the work of Kidd (7) wherein he attributes the resting stage in moist seeds to a narcotic action of carbon dioxide. Perhaps this idea may be correlated with certain data on insects. Buddenbrock and Rohr (8) working with the walking stick, *Dixippus morosus*, found that the rate of breathing movements is affected by the carbon dioxide content of the air. With increasing concentrations from 0 to 1 per cent. the rate of breathing suddenly increases, this is followed by a decrease; again at 10 to 15 per cent. there is a gradual increase; at 25 to 30 per cent. a decrease follows and at 35 to 40 per cent. respiratory movements cease. In the present paper there was found to be little or no change in the carbon dioxide content of an enclosed space containing an animal, when the original content was from 75 to 100 per cent. carbon dioxide. This excess carbon dioxide undoubtedly produces a deep narcosis in the grasshopper.

It may be of interest to note that experiments have been carried out by the author using a modified Van Slyke (9) method for determining the carbon dioxide content of insect blood. It seems that the carbon dioxide content of grasshoppers' blood is lower than that of mammalian blood and is approximately 30 cubic centimeters CO_2 per 100 cubic centimeters of whole blood, although sufficient data have not as yet been collected to state this exactly.

It would be of some importance to know the proportion of carbon dioxide that exists in the blood as free carbonic acid since the hydrogen ion concentration of the blood would be affected by varying carbon dioxide tensions unless a strong buffer system were present. With this idea in mind, a colorimetric hydrogen ion concentration determination on the same principle as that described by Cullen (10) was made and seems to indicate that the normal blood of *Melanoplus differentialis* is near a pH of 6.1.

SUMMARY.

1. It appears that exposures of *Melanoplus differentialis* and *Melanoplus femur rubrum* to a stream of pure carbon dioxide for various lengths of time up to about 30 to 40 minutes produce reversible effects. Further, the time to the first movement and to recovery after short exposure seems to be independent of the length of exposure. Longer exposures to the gas require longer periods for recovery.

It follows that the rate of the first movement and of recovery per minute exposure to the gas, up to 30 to 40 minute exposures, decreases rapidly. Above this limit the decrease in rates is imperceptibly gradual or remains constant until the exposure is sufficiently long to kill.

2. At 5 to 24 hours exposure to pure carbon dioxide, the animals are irreversibly affected and finally die. They may or may not exhibit reflexes upon removal from the gas.

3. Characteristic behavior, due to the influence of carbon dioxide, is exhibited.

4. There are three interdependent factors influencing the exchange of gases between the animal and the air in an enclosed space, namely, the original per cent. of carbon dioxide; the original per cent. of oxygen; the original ratio of carbon dioxide to oxygen.

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BIOLOGICAL BULLETIN

STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES: II. FEMALES OF *CAMBARUS VIRILIS* WITH MALE SECONDARY SEXUAL CHARACTERS.

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During the spring and summer of 1922 and 1923 the writer discovered nineteen specimens of *Cambarus propinquus* that possessed to some degree secondary sexual characters of both sexes. A close examination revealed that all were functional females, some of them bearing eggs when taken but possessing in addition to the usual female secondary sexual characters from one to three male secondary sexual characters as well. A number of males were found bearing extra male characters and two females with extra female characters. These results led to the examination of a fairly large collection of *Cambarus virilis* which had already been made and also to the search for aberrant conditions whenever specimens came to hand.

Next to *Cambarus propinquus Cambarus virilis* is the most abundant crayfish in southern Wisconsin and the two are usually taken together in the lakes and streams.

Intersexes in *Cambarus virilis*.

Specimens from Delavan Lake.

Intersexes in *Cambarus virilis* were first found in Delavan Lake on June 10, 1923. Subsequent collecting trips were made on June 11, June 14, June 30 and November 20. On June 10 fifty-seven mature females were taken, of which thirty-eight were carrying eggs. Thirty-seven of those carrying eggs had in addition to a full complement of female secondary sexual characters copulatory hooks on the third segments of the third walking legs as fully developed as those of the normal males (Fig. 1, *A*, *B*, *C*).

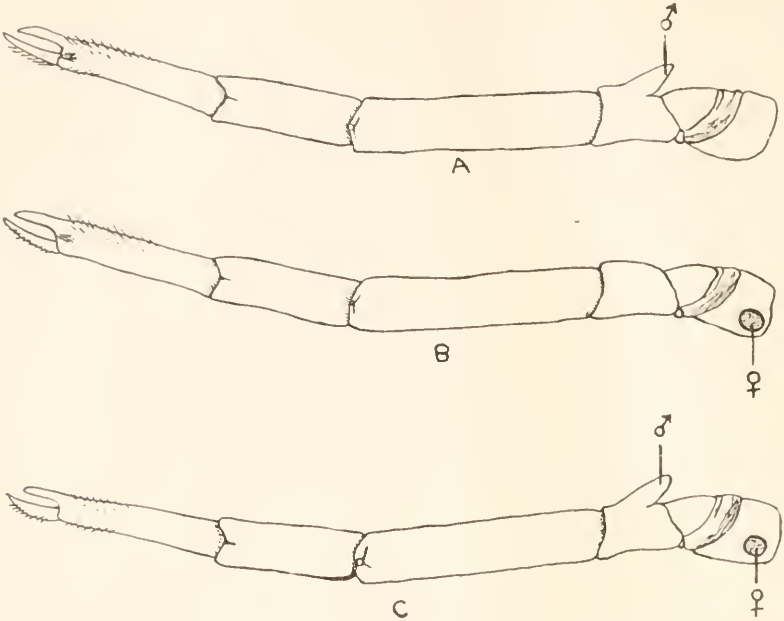

FIG. 1. Diagram illustrating the male copulatory hook on the third walking legs of a female.

A. Normal male walking leg with male copulatory hook. ♂.

B. Normal female walking leg with oviducal opening. ♀.

C. Intersex condition with both copulatory hooks and oviducal openings. ♂♀.

One of the specimens carrying eggs was a normal female. Of the remaining eighteen which did not carry eggs fourteen had well-developed hooks on one or both third walking legs. A number of specimens under 34 mm. in length were taken but they were too juvenile to have the hooks developed.

On June 11 six additional females were taken, three of which carried eggs. All possessed the copulatory hooks of the normal male and one large specimen had in addition a second set of secondary sexual characters in the form of small but distincly male-like appendages which replaced the usual rudimentary female appendages of the first abdominal segment (Fig. 2, *A*, *B*, *C*). The appendages resembled rather closely the normal appendages in their proximal halves but the usual slender tips of the normal male appendages were lacking and the appendages were only about half normal size.

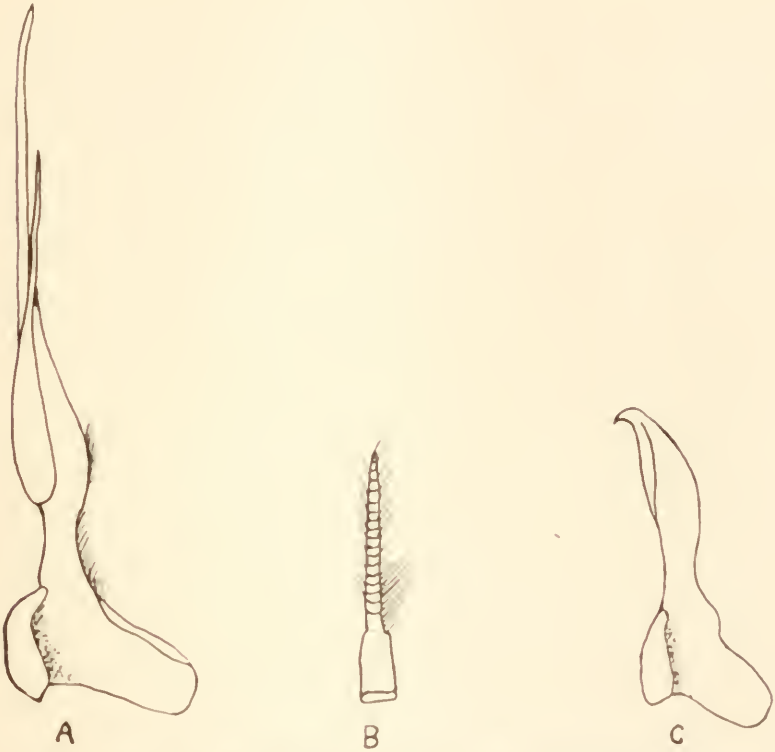

Fig. 2. Diagram illustrating the character of the first abdominal appendage in one of the intersexes.

A. Normal male appendage of the first abdominal segment.

B. Normal rudimentary female appendage of first abdominal segment.

C. Small but male-like appendage occurring on the first abdominal segment of one of the intersexes.

On the subsequent dates specimens were collected from various parts of the lake with results agreeing essentially with those first obtained. In all, over one hundred aberrant females were preserved and many more were examined and recorded. Upon the basis of the data gathered it was estimated that 88 per cent. of all the females of *Cambarus virilis* in the lake possessed at least one set of male secondary sexual characters.

Over two hundred males of both form I. and form II. have been examined and all have displayed only normal secondary sexual characters.

Specimens from Turtle Creek.

Since Turtle Creek forms the only outlet of Delavan Lake special attention was given to this creek and collections were made from the creek at three localities about eight miles apart. Of 263 females of *Cambarus virilis* collected a single one had the copulatory hook on the left third walking leg and was otherwise normal.

Specimens from Ponds.

A collection which was made in the spring and summer of 1922 from the ponds about Beloit was examined and out of 309 specimens (137 females) one female was found which had a copulatory hook on the left third walking leg.

Specimens from Rock River tributaries exclusive of Turtle Creek.

Collections were made from four small tributaries of Rock River, all within a radius of twenty miles and all females taken were normal. A collection of eighty females taken from Sugar River, a larger tributary of the Rock River, contained two aberrant female specimens each of which bore a copulatory hook on one of the third walking legs.

Specimens from Northern Wisconsin.

A small collection purchased from a supply by the University of Wisconsin contained sixty females two of which had copulatory hooks on both third walking legs. Through correspondence with the supply company it was learned that the specimens came from somewhere in northeastern Wisconsin.

Summary of Females with Male Characteristics.

1. In Lake Delavan 88 per cent. of the females of *Cambarus virilis* displayed at least one set of male characters, namely, male copulatory hooks on the third walking legs.

2. An old female from Lake Delavan carried in addition to the copulatory hooks a pair of small but distinctly male-like appendages upon the first abdominal segment.

3. Outside of Lake Delavan six females have been collected which had copulatory hooks on one or both third walking legs.

These aberrant females totalled about 1.5 per cent. of all the females collected outside of Lake Delavan.

Discussion.

From the data cited above it is evident that the presence of male secondary sexual characters in females of *Cambarus virilis* is not uncommon in the waters of Wisconsin. The exploration so far has been very limited but from the evidence already gained it seems quite probable that other localities may be found which will contain as large a proportion of aberrant females as have been found in Lake Delavan. It may be that sex intergrades in *Cambarus virilis* may be much more common generally than is supposed to be the case.

The small number of intersexes in *Cambarus propinquus* and in *Cambarus virilis* outside of Lake Delavan makes it possible to approach the question as to the possible causes for the intersexes in an indirect and hypothetical way but the very high proportion of intersexes in *Cambarus virilis* found in Lake Delavan should afford a much more satisfactory basis for such a study.

After observations and experiments upon intersexes in Fiddler Crabs Professor Morgan[1] reviews the possible causes for intersexes in Arthropods in general and more particularly in the Crustacea. He points out six possibilities. (1) The intersexes may be true hermaphrodites, possessing to some degree both ovaries and spermaries. (2) Parasitism affecting the host directly may be responsible for the intersexes. (3) Parasitism of the gonads may cause a loss of the supposed control of the direction of development of the secondary sexual characters. (4) The intersex condition may be due to some environmental influence. (5) Changes in genetic constitution may account for the condition. (6) Peculiar or accidental embryonic development may be the cause.

Of the above possibilities the third and the fifth have been considered in connection with intersexes in *Cambarus propinquus* in another paper.[2] It was borne in mind that the evidence obtained thus far in the Arthropods does not favor the assumption of control of the direction of development of the secondary sexual characters by secretions from the gonads. However, the prevalence of parasitism in the forms studied could not be overlooked and parasitism of the gonads was stated as a possible cause for the intersex condition. More weight was attached to the possibility of genetic disturbances being the important factor for reasons to be stated later. In the aberrant specimens described here parasitism was variable in its occurrence among the intersexes even to the point of being non-existent in a considerable number. Parasitism, either as affecting the body directly or the secondary sexual characters indirectly through the gonads cannot, therefore, be considered seriously.

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[1] Morgan, T. H., 1920, *American Naturalist*, Vol. 54, pp. 220–246.

[2] Turner, C. L., 1924. "Studies on the Secondary Sexual Characters of Crayfishes. I. Male Secondary Sexual Characters in Females of *Cambarus propinquus*," Biological Bulletin, Vol. XLVI., No. 6.

If the specimens described here were truly hermaphroditic it should be possible to find some traces of testicular tissue within or near the ovaries. All specimens seem to have been functional females and at a season when they might be expected to carry eggs or embryos they were doing so. A considerable number of specimens have been carefully dissected and macroscopic and microscopic examinations made but with the result of finding nothing that might be called testicular tissue. Testicular tissue may be present but it has not been found as yet.

The possibility that some environmental influence such as peculiarities of temperature or chemical substances in solution may have been causing the aberrancies can be tested by removing specimens from Lake Delavan while they are still in the egg stage and rearing them under changed conditions. Such a study is on the program for the coming spring.

Peculiar or accidental embryonic development without attendant environmental influences to account for them might be considered to account for a small number of intersexes but this factor could scarcely be invoked to account for as large a proportion as 88 per cent.

The suggestion that changes in genetic constitution have been the underlying cause seems by the process of elimination to be the logical choice and indeed there is considerable direct evidence to support such a claim. This possibility was suggested in the cases of *Cambarus propinquus* previously described for the reason that intersexes from the same localities showed a remarkable degree of similarity from which it could be inferred that they might have been members of the same brood. The evidence from Lake Delavan for the inheritance of the intersex condition lies in the fact that at least three generations of crayfishes were represented in the specimens secured. If the peculiar condition were being transmitted it should be expected that the same proportion of intersexes would obtain in specimens representing different ages. This was found to be the case. Admitting that constant but peculiar environmental conditions might be a possible cause it is strongly suspected that the cause for the intersex condition lies in some change in the genetic constitution, possibly in the chromosome complex and that the condition is being inherited. It is proposed to test this inference by collecting intersex females which are bearing eggs and to rear the eggs to maturity or to such a time as it will be possible to determine whether or not the young possess the peculiarities of the mother.

STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES: III. MALES WITH SUPERNUMERARY MALE SECONDARY SEXUAL CHARACTERS IN THE SUBGENUS *FAXONIUS.*

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In the genus *Cambarus* the males of the subgenus *Cambarus* are distinguished from the males of the other subgenera by the presence of copulatory hooks upon the third segments of the third and fourth walking legs. The other subgenera possess these copulatory hooks only upon the third walking legs. These hooks are secondary sexual organs and are used to some extent in the process of copulation. It has been supposed that the number was quite constant and the few specimens that have varied from the normal condition have been classed as "freaks." Ortmann[1] in discussing the use of the copulatory hooks as diagnostic features says of *Cambarus virilis* and of *Cambarus propinquus* ". . . always only the third pereiopods with hooks, barring freaks."

A large number of individuals, indeed, enough to constitute a considerable proportion of a crayfish population have been found in *Cambarus virilis* and in *Cambarus propinquus* to deviate from this rule in the possession of more than one pair of hooks. These specimens can scarcely be classed as freaks. The deviation becomes the more interesting since it occurs in a crayfish population which has a large proportion of other aberrant conditions in the secondary sexual characters such as the occurrence in females of male secondary sexual characters, the occurrence in males of female secondary sexual characters and the occurrence in females of extra female secondary sexual characters.

The thirty-two specimens to be described have been taken with the exception of three from the lakes and streams of Wisconsin,

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[1] Ortmann, A. E., 1905, "The Mutual Affinities of the Species of the Genus *Cambarus* and Their Dispersal Over the United States," *Proc. Amer. Phil. Soc.*, Vol. 44, pp. 91 136.

It seems likely that the survey need only be extended to discover many more specimens since the present ones were taken in a very limited bit of collecting.

Description of Specimens.

1. *Specimens of Cambarus virilis.*

Two specimens have been taken from the tributaries of the Rock River in the vicinity of Beloit, Wisconsin, both bearing in addition to the copulatory hooks of the third walking legs a second pair of such hooks on the second walking legs. The additional hooks were slightly smaller than the normal ones but were distinct. Both specimens were about two years old. They represented a proportion of about .5 per cent. of all the males taken.

A collection of large specimens examined at the University of Wisconsin and later traced through a supply company to northeastern Wisconsin as the locality from which the collection came contained forty-eight males of which sixteen had copulatory hooks upon the second walking legs in addition to those of the third walking legs. Two other males in this same collection had each a single additional hook upon the left second walking leg. The additional hooks were in some cases as well developed as those normally occurring upon the third walking legs but in most instances they were smaller. In this same lot of specimens there were 3.5 per cent. of females which possessed each a well-defined male characteristic in the form of a pair of copulatory hooks on the third walking legs.

2. *Cambarus propinquus.*

In this species the occurrence of males with additional male characteristics has been found to be rather constant but in no locality has there been so large a proportion of aberrant individuals as in *Cambarus virilis*. There is, however, a greater variation in the additional characters. In some instances the additional hooks are found upon the fourth walking legs and in others upon the second walking legs.

During the summer of 1922 considerable numbers were collected from time to time from Turtle Creek but only two males were noticed which had extra copulatory hooks. In one instance the additional hooks were carried upon the fourth walking legs and in the other upon the second walking legs.

In July, 1923, a collection was made from a tributary of Turtle Creek twelve miles from the first locality. Of 150 males five had additional copulatory hooks. Three of these had well developed additional hooks upon the second walking legs while the other two specimens bore the extra hooks upon the fourth walking legs.

A single specimen from a collection of twenty-five males from Delavan Lake had extra copulatory hooks upon the fourth walking legs.

A collection of 152 male specimens from a tributary of Rock River twelve miles south of Turtle Creek contained three with extra male hooks, two of them bearing the extra hooks upon the second walking legs and the other bearing them upon the fourth walking legs.

3. *Cambarus obscurus.*

A small collection of thirty-eight males from Ashtabula, Ohio was examined at Ohio State University and one male was found with extra copulatory hooks upon the second walking legs.

4. *Other Species of Cambarus.*

An examination of 1280 specimens from eight additional species of *Cambarus* failed to bring to light any new aberrant specimens.

Discussion and Summary.

The records cited above are quite limited but a few general facts become apparent when they are examined critically.

1. Supernumerary male secondary sexual characters seem to differ with the species in which they occur. An examination of 600 specimens of *Cambarus clarkii* failed to reveal any deviations from the usual male secondary sexual characters either in the copulatory hooks upon the third and fourth walking legs or in the modified appendages of the first and second abdominal segments. An examination of 310 specimens from three species of the subgenus *Faxonius* likewise failed to show any aberrant features. On the other hand there is a rather constant occurrence of additional copulatory hooks in *Cambarus propinquus* as represented by specimens from southern Wisconsin, northern Illinois

and northern Ohio. About 2 per cent. of all the males of *Cambarus propinquus* examined have been aberrant in this respect. In *Cambarus virilis* the general occurrence of specimens with supernumerary sexual characters is less but locally the proportion of specimens abnormal in this respect mounts up to 33 per cent.

If the cause or causes underlying the peculiar condition is to be studied to advantage it would be in localities in which the proportion of peculiarites was remarkably high.

2. The manner in which specimens with supernumerary hooks vary from the normal seems to have a certain degree of constancy in a given species. In *Cambarus virilis*, for example, the deviation is always in the direction of the development of additional hooks upon the second walking legs. In *Cambarus propinquus*, on the other hand, the additional hooks may be developed upon either the fourth or the second walking legs but never upon both the fourth and the second walking legs in the same specimen.

3. Deviation has thus far always been in the direction of more than the normal number of copulatory hooks and not less than normal. This has held for all species examined whether they normally possess copulatory hooks upon the third and fourth walking legs, as in the case of *Cambarus blandingii* acutus and *Cambarus clarkii*, or upon the third walking legs only as in all species of the subgenus *Faxonius* and the subgenus *Bartonius*.

4. It is suggested that, in *Cambarus propinquus*, since the second, third and fourth walking legs are potential bearers of copulatory hooks a specimen may be found expressing the extreme degree of deviation from the normal condition and bearing copulatory hooks upon all three of these walking legs.

FORMATION OF FOOD-CUPS IN *AMŒBA* INDUCED BY CHEMICALS.

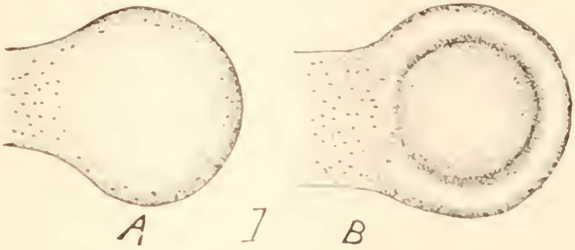
J. GRAHAM EDWARDS.

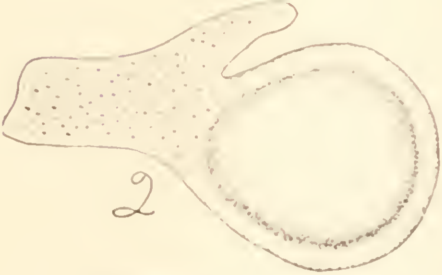
From the Zoölogical Laboratory, The Johns Hopkins University.

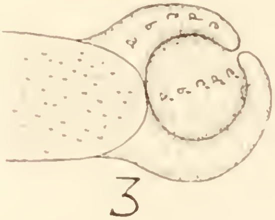
Feeding reactions of *Amœba* have been described by different investigators and from various standpoints. Jennings (1), Kepner and Taliaferro (3), Mast and Root (4) Schæffer (5), and Beers (6) have contributed valuable data concerning such reactions. Jennings mentions in connection with the extension of a pseudopod toward a food particle, that this should be attributed partly to chemical stimulation. Mast and Root hold that surface tension is probably an insignificant factor in the process of feeding, and Schæffer is of the opinion in this connection that the presence alone of a substance in solution is not sufficient to attract *Amœba*, or to cause ingestion, but that the substance must be actively diffusing from a definitely localized region. Kepner and Edwards (2) conclude that there is no hypothesis as yet advanced to explain quantitatively the reaction involved in the movement of Rhizopoda, which can be applied to *Pelomyxa's* movement around food-bodies.

While locomotion and movements observed in feeding still remain unexplained, the following observations of feeding reactions induced by chemicals may further extend or limit this problem. For feeding reactions quite comparable in every way to those described by the investigators mentioned, occur without the presence of food and in a homogeneous medium. There are no visible stimuli.

When amœbæ are washed in three changes of distilled water and immersed in certain chemically pure salt solutions, food-cups are formed in the absence of solid particles which are comparable to those observed in the process of ingesting food. These cups are formed as follows: On the under surface of one or more pseudopods a local expansion of the ectoplasm occurs accompanied by an inflow of endoplasm. Immediately the marginal surface of the protuberance formed by this local expansion becomes firmly attached to the substratum and the enclosed area arches upward causing much of the endoplasm between the upper and lower surface to recede: thus forming a spoon- or cup-like concavity. The marginal surface of this concavity draws slowly together from all directions reducing the aperture of the concavity until it is entirely closed. A vesicle is thus formed not unlike the







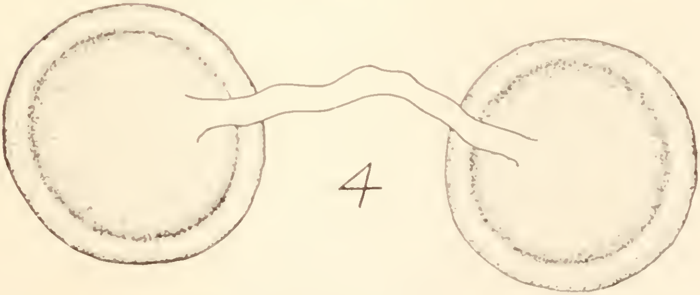


FIG. 1, *A* AND *B*. Free hand sketch of the pseudopod of an *Amœba* during the formation of a food-cup.

FIG. 2. A similar sketch of a food-cup which involves almost the entire *Amœba*.

FIG. 3. Another type of food-cup formation in which two accessory pseudopods arise and advance consecutively through positions indicated by *a*, *b*, *c*, *d*, *e* followed by an overarching sheet of ectoplasm indicated by a^1, b^1, c^1, d^1, e^1.

FIG. 4. In this type of food-cup formation the two cups formed are connected by a strand of protoplasm.

contractile vacuole in appearance (Figs. 1, 2, 3 and 4). This vesicle is then constricted off from the ectoplasm and borne along in the endoplasmic current as a normally formed food-vacuole. The size of the concavity varies depending apparently on the size of the pseudopod giving rise to it. If the amœba is monopodal, the concavity and vesicle are large; if heteropodal, they are smaller. The upper as well as the lower surface may develop such a concavity and vesicle so that contact with the substratum is not necessary. The food-cups thus formed rarely remain visible for longer than 3 to 5 minutes.

The most favorable media for inducing these feeding reactions are as follows: A mixture of NaCl, LiCl and $CaCl_2$; NaCl alone or mixtures of neutral sodium salts; $CaCl_2$ and a mixture of NaCl with $CaCl_2$; or $NaNO_3$ mixed with $Ca(NO_3)_2$. Mixtures of the different salts used were always of the same concentration and in equal parts. Suitable concentrations are $N/300$ or $N/500$. No appreciable difference exists in either concentration as to the size or number of food-cups formed. Sea-water diluted by adding two parts of distilled water to one of sea water is also an excellent medium for such feeding reactions. Less favorable media are KCl, $N/500$, and mixtures of potassium salts, $MgCl_2$ and $MgSO_2$. In neutral ammonium salts and in $SrCl_2$ and $BaCl_2$, $N/500$, these reactions occur only occasionally.

In the most favorable mixture, *i.e.*, NaCl, LiCl and $CaCl_2$, containing a hundred or more amœbæ, this reaction is observable within one or more hours in a considerable number of specimens and thereafter almost continuously in now one group, now another. In a few hours it is possible to obtain as many amœbæ as desired in all of the stages manifest in this reaction. Owing to the rigidity of the marginal surface of the food-cup, amœbas can be removed from the solution with a pipette and immersed in a fixing fluid without appreciable change in the character or form of the food-cup. Examination of food-cups when amœbæ are in the solution as well as after they are fixed and sectioned fails to show the presence of any particle or other visible stimulus normally present in feeding reactions.

Feeding reactions also occur in a dilute acid or base, *e.g.*, HCl or NaOH if a neutral salt, *e.g.*, NaCl is added. Feeding reactions were not observed to occur in other than neutral salt solutions except to a slight extent in $NaHCO_3$. It is this condition that shows the probable significance of the pH of the medium in conditioning such reaction and the effect of the medium on the surface. In general, solutions impeding or preventing locomotion do not induce feeding reactions.

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THE STRUCTURE OF THE UNDULATING MEMBRANE IN THE CILIATE *BLEPHARISMA*.

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It has for long been generally accepted [1] that the undulating membrane of ciliates is composed of a serried row of fine cilia fused into a homogeneous-appearing membrane. This conception has been based largely on the fact that the membrane, when treated with fixatives, resolves itself into a linear series of very fine fibrillæ. Moreover, several investigators have remarked on the occasional appearance of delicate striations in the living membrane of certain ciliates. In addition to this Maier reports having noticed, in the living specimen an actual breaking down of the membrane into fibrillæ for some distance inward from its free border.

It was thought of interest to study the behavior of the undulating membrane in *Blepharisma undulans* by subjecting it to manipulaton with the microdissection needle.

A single blepharisma, in a shallow hanging drop in a Barber's moist chamber, was held stationary by means of a microneedle thrust into the posterior end of the body, Fig. 1, *a*. Another needle, Fig. 1, *b*, having a very slender and long tapering tip (the

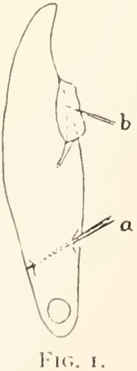

Fig. 1.

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[1] For a review of the literature on this subject see H. N. Maier, "Der feinere Bau der Wimperapparate der Infusorien," *Arch. f. Protistenk.*, 2, 73 (1903).

needle being about one micron in diameter at about five micra back from the tip) was carefully brought up to the free margin of the undulating membrane. By this means the membrane could be considerably deflected without being torn. The undulations continued in spite of the forced position of the membrane.

With the needle it was found possible to press down upon the membrane and to pierce it, Fig. 2, *a*. The membrane at once split vertically along a line running through the puncture, Fig. 2, *b*. The part of the membrane beyond the needle then rapidly broke into a linear series of very fine cilia which were plainly visible because they were now beating out of unison Fig. 2, *c*. As long as the needle was kept in position the cilia of the disrupted portion of the membrane repeatedly fused and separated again. The fusion was partial, being limited to several groups of the cilia which repeatedly kept fusing and then breaking apart.

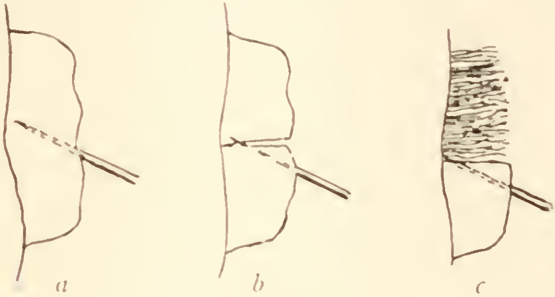

FIG. 2.

When the needle on the membrane was removed the entire row of cilia quickly fused and reconstituted the membrane. By less delicate treatment with the needle the entire membrane could be broken up momentarily into cilia. Upon removal of the needle the cilia always reunited to form an intact, homogeneous membrane. A membrane could thus be repeatedly disrupted and then allowed to resume its original normal aspect without any sign of having been injured. This is indicated by the fact that when the animal is freed by removing the transfixing needle it will swim away with the membrane undulating normally.

A temporary, partial breakdown of the undulating membrane was also observed to occur spontaneously while under dark-field illumination. The picture was most striking for the membrane, while intact, could be detected only because of its shimmering and silvery outline. Suddenly, the membrane was seen to break up into innumerable cilia beating out of unison. A few seconds later the brilliantly lighted cilia disappeared as they fused into an optically structureless membrane.

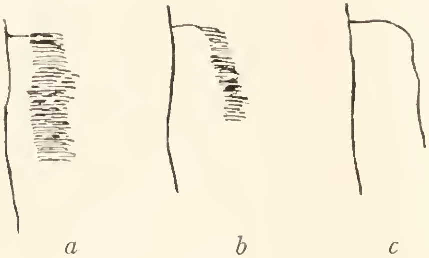

FIG. 3.

It is significant that the reconstitution of the membrane occurs by a fusion which spreads from the bases of the cilia toward their free ends. Three successive stages of this process are shown in Fig. 3, *a*, *b* and *c*. This suggests the possibility of a slime-like substance being secreted and spreading between and over the cilia to join them into an optically homogeneous sheet.

THE EYE AND OPTIC TRACT IN NORMAL AND "EYELESS" *DROSOPHILA*.

MILDRED HOGE RICHARDS AND ESTHER Y. FURROW.

(Contribution from the Zoölogical Laboratory, University of Oklahoma, Second Series, No. 48.)

One of the most interesting mutations that has occurred in *Drosophila melanogaster* is known as "eyeless." It was first observed by the senior author in 1913, when some flies with eyes entirely wanting on one or both sides appeared in the cultures. The inheritance of eyeless was worked out at that time and the gene which is responsible for the condition was located in the fourth chromosome. The stock breeds true to the eyeless condition, but there is no fixed type of eyeless. It seems very probable that while the known gene in the fourth chromosome determines that the eye shall be reduced, other genes not yet located govern the extent of the reduction of the eye.

Recently Little and Jones have carried on a selection of eyeless flies in order to obtain races showing large and small proportion of eyeless. Some progress was made and races of these two kinds were separated. These results may indicate a genetic difference between the two lines.

The name "eyeless" was perhaps incorrectly applied, since all the flies of the pure stock are not entirely without eyes. There is considerable variation in the size of the eye. Zeleny has found that the eye of a normal fly of certain strains which he had under investigation contains from 800 to 840 ommatidia. In the eyeless stock the eye may be totally wanting, or it may appear superficially to be normal in every particular except size, even at its maximum being smaller than the eye of the normal wild type. Between these two extremes all gradations appear. Furthermore there is usually in eyeless an entire lack of symmetry between the eyes of the two sides. Sometimes the eyes are about equal in size, sometimes present on neither side, and sometimes there will be a fair sized eye on one side while on the other there will be only a small one or none at all.

A superficial examination of the heads of eyeless flies revealed the fact that those totally eyeless have no external trace of ommatidia. An interesting question, first suggested by Dr. T. H. Morgan, arose concerning the condition of the optic tract in the totally eyeless flies. The heads of such flies are greatly reduced in size resembling pin heads. Since the eye is gone and the head so greatly reduced, it would seem highly probable that a large part of the optic tract may be wanting. Accordingly a comparative study of the heads of normal and eyeless flies was undertaken. Sections were cut and a special study made of the optic tract. No attempt was made to study the brain nor was the optic tract studied neurogically. This had been done for the bee by Kenyon. The problem was to compare the optic tracts of normal and eyeless strains. In other words what part of the optic tract is missing in eyeless?

Technique.

The flies were anæsthetized with ether. Then with the aid of a lens the heads were cut with a razor blade from the body so as to include most of the thorax as well as the head, since it was found that subsequent handling was considerably facilitated by the presence of the thorax. The desired portion thus cut off was immediately immersed in the killing fluid. Several killing fluids were tried but the most satisfactory was Bouin. Any picric acid combination proved satisfactory, and considerable success followed the use of a warm saturated solution of picric acid in 50 per cent. alcohol. Material was allowed to remain in Bouin 24 hours. It was then dehydrated and embedded in paraffin. Sections were cut at various thicknesses, 5, 7½, 10, and 15 microns. For general study the thickness of 10 microns was most practical. Both hæmalum and iron hæmatoxylin were used as stains. For detailed work the latter was more satisfactory and was used extensively throughout the study. In a few cases eosin was used as a counterstain.

Description.

The eye of *Drosphila* is very large and occupies almost the whole side of the head (Plate III., Fig. 1). Our sections revealed the structure of the eye. It is of the large compound type characteristic of insects. The whole eye with its most distal ganglion, called in this paper the outer (periopticon of Hickson) has in general the shape of a cone of rather low altitude with the apex directed inwards. The surface of the eye is covered with small facets between which project hair like bristles. (Plate I., Fig. 1, and Plate III., Fig. 2.) Each facet is the outer portion of a single ommatidium. In the center of the eye the ommatidia are at right angles to the basement membrane but at the sides of the eye they join the membrane at a considerable angle. Beneath the layer of facets is a layer of pseudocones or lens-like structures. In the preparations there is often an artificial separation between the facets and the pseudocone layer. Immediately under the pseudocones there is a layer of retinulæ and rhabdomeres. The latter are darkly stained rods which stand out clearly in our sections. Under this layer is the basement membrane which separates the eye from the outer ganglion.

A closer study of the eye under a higher magnification reveals more clearly the nature of each ommatidium. Each pseudocone appears as a hollow space on whose proximal side are two of the four cone cells which produce it, with their contained nuclei (Semper's) (Plate I, Fig. 2). The pseudocone is characteristic of flies with short antennæ, and is in contrast with the eucone in which the nuclei are distal to the lens. Beneath each pseudocone is a spindle-shaped group of rods or rhabdomeres. Outside each rhabdomere is an elongated cell or retinula. Outlines between the retinulæ do not show clearly in most of our longitudinal sections, but between retinulæ of adjacent ommatidia, pigment cells can be seen. At the ends of the spindle the rhabdomeres are close together, while at the middle of it they are farther apart. In each ommatidium there are seven retinulæ and seven rhabdomeres. Six of the latter are approximately equal in size, but the seventh is smaller and seems to be joined edgewise to a small lamella. Plate I., Fig. 3 shows a cross section of an ommatidium near the distal end. Here the seven rhabdomeres are close together, and the outlines of the seven retinula cells, some of which show nuclei, can easily be made out. Plate I., Fig. 4, shows a cross section of the ommatidium proximal to that of Fig. 3. Here the rhabdomeres are farther apart and the spindle-shaped ommatidium has widened. The retinulæ are much smaller in section and appear as a protoplasmic circle around the rhabdomeres. Indentations in this protoplasmic ring reveal the position of the boundaries of the retinulæ. Each retinula has, as the cross sections indicate, a nucleus at the distal end just below the nucleus of the cone cell. Nuclei were also observed at the proximal end of the retinulæ, although we were not able to determine with accuracy whether each one has a nucleus at this level.

Pigment cells separating the ommatidia were distinguished in both longitudinal and cross sections. These separate the cone cells and the retinulæ of the neighboring ommatidia. Since the pigment cells did not seem to have a practical bearing on our problem, no attempts were made to demonstrate them with special methods by which a more complete study of them would be possible.[1] Hence the drawings do not include them.

The eye is connected to the brain by a series of three ganglia which we will call the outer, median, and inner; these correspond to the same ganglia named by Hickson from the outside inward as periopticon, epiopticon, and opticon respectively. The outer ganglion joins at its distal edge the basement membrane and is flattened out to conform to the shape of the eye. Centrally it narrows passing into nerve fibers which decussate to enter the median ganglion (Plate II., Fig. 1). The median and inner ganglia have each the shape of a meniscus lens in transverse sections. From the median ganglion fibers pass to the inner. The inner ganglion is connected to the brain by short fibers which do not seem to cross. There is thus no optic nerve as in crustaceans, a fact long ago pointed out by Berger.

A more detailed study of the outer ganglion (Plate I., Fig. 5) shows that it consists in its proximal part of nerve fibers arranged in groups in parallel fashion. These groups constitute the elements of the outer ganglion. In the distal part among cells which do not seem to be uniformly arranged are scattered tracheal vessels and groups of fibers which reach to the basement membrane. The connections between these fibers and the elements of the ganglion could not be followed in our sections.

When the heads of eyeless flies were studied certain facts became at once evident. Many of the eyes of the eyeless stock were simply reduced in size. Most of these reduced eyes have a large number of ommatidia (Plate II, Fig. 2), but obviously even the larger eyes have a smaller number than have the normal. Some of the eyes, however, are very small, sections through the middle showing scarcely half a dozen ommatidia. Plate III., Figs. 3 to 8 are microphotographs through heads of eyeless flies. In Fig. 3 there is a small eye on the right side but none on the left. On the right side the three optic ganglia are present, for a higher magnification reveals the fact that the middle ganglionic mass here shown is duplex, representing both the median and the inner ganglia. Fig. 4 shows a head with a small eye on each side. On the left side the outer ganglion is somewhat rounded, and the mass of fibers connecting it to the median ganglion is larger in proportion than that of the normal eye. Below this mass of connecting fibers may be seen the median and inner ganglia, each a double lens-like structure as in the normal eye. The section does not go through the optic tract of the right side. Figs. 5 and 6 show each a fair-sized eye on the right side and also the three ganglia of the right optic tract. In Fig. 5 the section goes through but a small portion of the brain. Fig. 7 shows a smaller eye on the right side and all three optic ganglia, although in the picture the median and inner are so close that they seem to form a single mass. These and other sections show that where the ommatidia are present at all they seem to have the normal structure and all three of the optic ganglia are present though in many cases they are reduced in size corresponding to the size of the eye.

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[1] While this paper was in proof, a study by O. A. Johannsen, entitled "Eye Structure in Normal and Eye-Mutant Drosophilas" appeared: Jour. Morph. Vol. 39, no. 2. Dec. 1924. The pigment cells are treated in detail in this work.

In the totally eyeless flies, that is, in those flies that showed no external trace of ommatidia, the outer ganglion was also absent (Plate II., Fig. 3). This most distal ganglion had disappeared as had the eye itself. The other two ganglia, however, were present, though much contracted and in close apposition. This is well illustrated by the text-figures 1 to 5 which are outlines of the median and inner ganglia in the totally eyeless condition. These two ganglia have become a rounded, more or less shapeless mass, and it is only with high magnification that the division between them can always be made out. A close study of the ganglia, however, always revealed the fact that the mass is duplex, representing both median and inner. Sometimes one of the ganglia, as in Fig. 3, will show the double lens-like structure best seen in transverse sections. In Fig. 4 each of the two ganglia show this double nature, similar to that of the normal eye. The contraction in the two inner ganglia in part explains the great reduction in the size of the eyeless head.

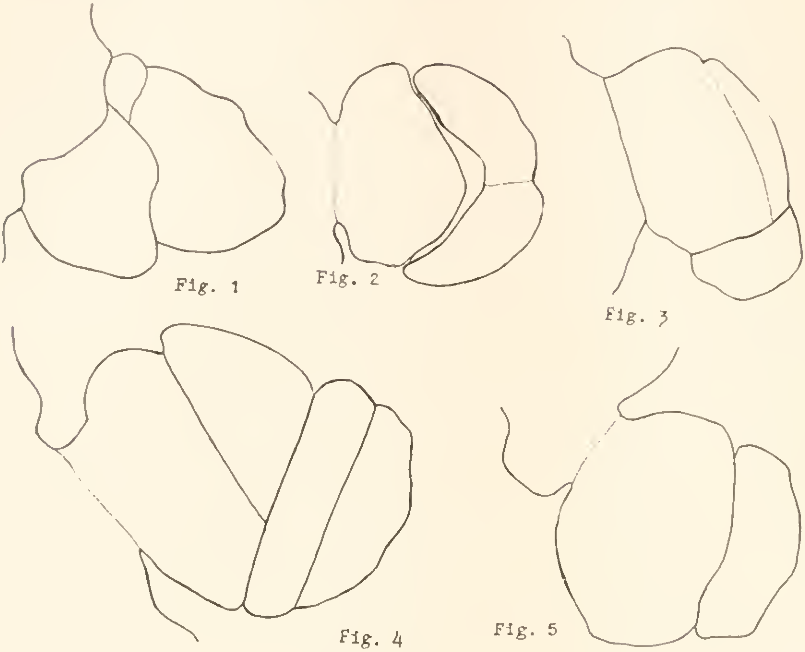
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5

FIGS. 1–5.

We have never failed in the many heads studied to identify in the totally eyeless half both median and inner ganglia. Plate III., Fig. 3, shows no eye on the left side, but here the section does not go through the optic tract. However, both the median and inner ganglia appear in other sections of this head. Fig. 5 shows also a totally eyeless condition on the left side and there is no trace of the outer ganglion. The section goes through the whole optic tract of this side showing clearly both median and inner ganglia, each having a double lens-like appearance as on the opposite side, where the eye is present. The left side of the head is correspondingly smaller than the right giving it a twisted appearance, and there

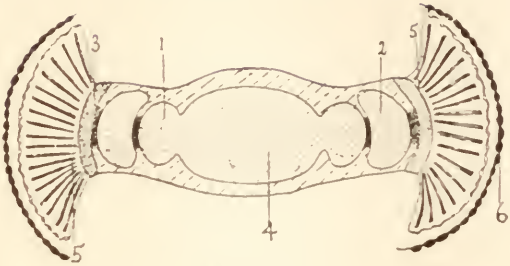

Fig. 6

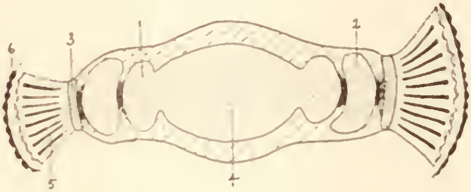

Fig. 7

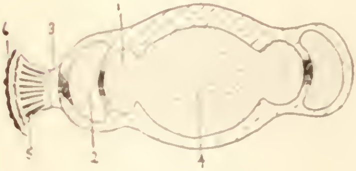

Fig. 8

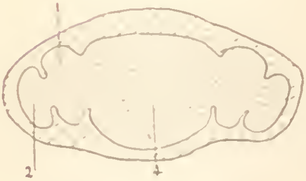

Fig. 9

FIGS. 6–9. Diagrammatic cross sections. FIG. 6, normal; FIG. 7, both eyes reduced; FIG. 8, left eye reduced, right wanting; FIG. 9, totally eyeless.

1, inner ganglion; 2, middle ganglion; 3, outer ganglion; 4, brain; 5, ommatidia; 6, facets.

is considerable difference in the size of the ganglia of the two sides. Fig. 6 shows a totally eyeless condition on the left side but here the section does not go through the optic tract. In Fig. 7 there is no trace of the eye on the left side, but the inner ganglion is shown here and the median in other sections. Figure 8 is a frontal section of a totally eyeless head with no sign of ommatidia on either side.

Text-figures 6 to 9 illustrate in diagrammatic fashion the condition of the optic tract in normal and eyeless flies. In Fig. 6 the eyes are normal and all three ganglia are present. In Fig. 7 the eyes are reduced but the three ganglia still remain. In Fig. 8 the left side repeats the condition of Fig. 7 but the right side is totally eyeless and only the median and inner ganglia remain. In Fig. 9 both sides are totally eyeless and in both the outer ganglion is absent.

DISCUSSION.

It is an interesting fact that the loss of the eyes causes the loss of only the outer ganglion, the two inner remaining, although in a modified condition. From its structure and form we would hardly expect the outer ganglion to remain when the eye is gone. It is so intimately connected to the eye throughout its whole distal surface by fibers which pass through the basement membrane that it is difficult to see how the eye could disappear without it. The other two ganglia have obviously a very different structure. Between either two of these three ganglia the separation might easily occur, but it is evident from the preparations that the break in the optic tract has taken place at the first point where a separation is structurally simple.

There has been considerable discussion in the past concerning the homology of the optic tract of insects. Berger first pointed out the comparison between the narrow constriction separating the brain and inner ganglion and the entire optic nerve of other forms. The retinular layer he regarded as homologous only to the rod and cone layer of the vertebrate eye.

Patten however regarded the cone cells as the percipient element and thought them connected to the rhabdome. Lowne conceived of the outer ganglion as the true retina and thought that no fibers pass from the retina through the basement membrane to the eye, but that the fibers ended in the outer ganglion; these conclusions seem not to have been confirmed by other workers. Most modern investigators agree with Parker, who has identified the rhabdomeres as the retinal elements, and has been able experimentally to produce upright images in them. His experimental conclusions are borne out by his detailed work (Crustacea) on the structure of the rhabdomeres as well as by that of Hesse. The latter has been able in insects to trace nerve fibrils passing from the rhabdomeres to the retinulæ, each retinula ending in a nerve fiber which goes through the basement membrane.

Since the rhabdome layer is to be regarded as the true retina it would seem that the optic nerve should properly be considered as distal to the ganglia rather than as between inner ganglion and brain according to Berger's conception. This is the view taken by Wheeler who regarded the optic nerve in ants as peripheral to the optic ganglion. The present work would seem to lend some support to the idea that the mass of decussating fibers between the outer and middle ganglia is the optic nerve, for when the eye disappears, it would be most natural to expect the break in the optic tract to take place at the optic nerve. It must be pointed out that we lack definite embryological information as to the origin of the outer ganglion, and until this is obtained, the suggestion just made can be only provisional.

SUMMARY.

Flies of the mutation eyeless (*Drosophila melanogaster*) have small eyes on both sides or are sometimes totally eyeless on either or both sides. The eye and the optic tract in normal and eyeless stocks were studied in order to determinate what part of the optic tract is lacking in the eyeless flies. In the normal fly, three ganglia, outer, median and inner, connect the eye with the brain. In flies with small eyes all three ganglia are present and the ommatidia are normal although greatly reduced in number. In totally eyeless flies the outer ganglion is missing and the median and inner are contracted into a more or less shapeless mass which nevertheless discloses its double nature.

Since the retina has been identified by modern workers as the rhabdome layer of the eye, these observations lend support to the idea that the optic nerve is a mass of fibers connecting the outer and median ganglia.

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DESCRIPTION OF FIGURES.

All drawings in this paper are by Esther Y. Furrow.

Abbreviations.

b—bristle.
bm—basement membrane.
c—cell layer.
df—decussating fibers between outer and median ganglia.
f—facet.
fb—parallel groups of nerve fibers.
ig—inner ganglion.
mg—median ganglion.
ng—nerve fibers reaching to basement membrane.
np—nucleus of pseudocone.
nr—nucleus of retinula.
og—outer ganglion.
om—ommatidium.
p—pseudocone.
r—retinula cell.
rb—rhabdomere.
rl—layer of retinulæ and rhabdomeres.
t—cut ends of tracheæ.

PLATE I.

FIG. 1. Section through the head of a normal fly. × 180 (16 mm. obj. 12.5 ocular).

FIG. 2. Longitudinal section through several ommatidia of a normal fly (1.9 obj.).

FIG. 3. Cross section of a single ommatidium of a normal fly near the distal end of the rhabdomeres. × 1500 (2 mm. obj., 12.5 ocular).

FIG. 4. Cross section of a single ommatidium of a normal fly more proximal than that of Fig. 3 (same magnification as that of Fig. 3.).

FIG. 5. Details of the outer ganglia (Transverse section of head). × 825 (1.9 obj., 7.5 ocular).

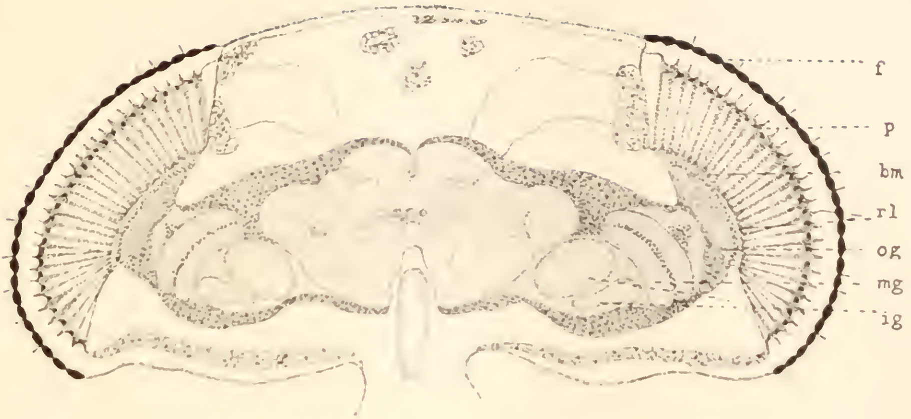
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Fig. 1

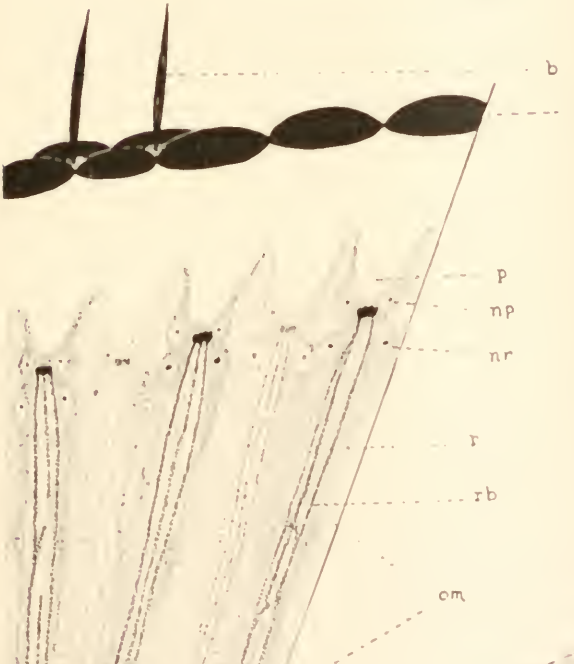
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Fig. 2

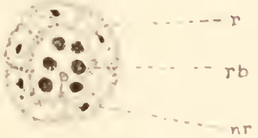

Fig. 3

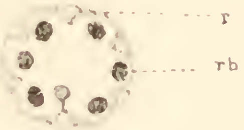

Fig. 4

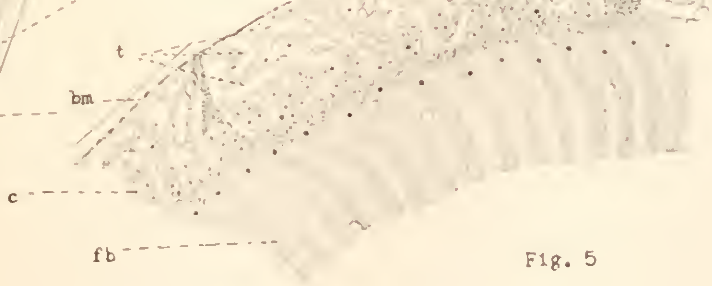

Fig. 5

Plate II.

Fig. 1. Details of the three ganglia (Transverse section of head). × 495 (4 mm. obj., 10 ocular).

Fig. 2. Transverse section of head of fly with small eyes. Same magnification as Plate I, Fig. 1.

Fig. 3. Transverse section of totally eyeless fly. Same magnification as Fig. 2.

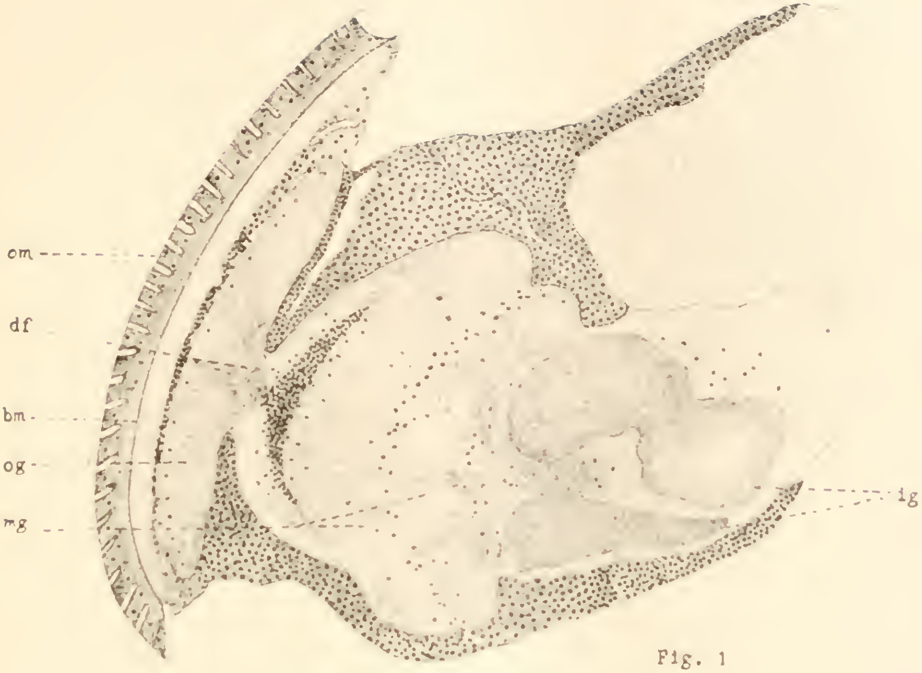
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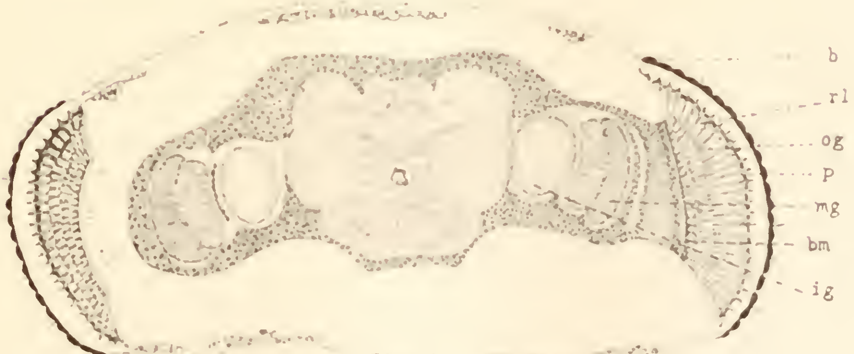
Fig. 1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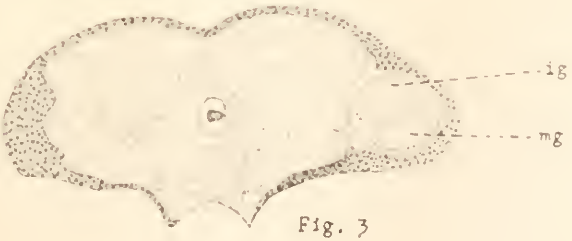

Fig. 3

PLATE III.

Microphotographs.

FIG. 1. Side view of head of living normal fly.

FIG. 2. Transverse section of head of normal fly, from slide loaned by Dr. F. Payne.

FIG. 3–7. Transverse sections of heads from eyeless stock.

FIG. 3. Head is totally eyeless on left side, has very small eye on right. Section does not go through optic tract of eyeless side.

FIG. 4. Head has two small eyes. Section goes through optic tract of left side.

FIG. 5. Head has slightly larger eye on right; is totally eyeless on left. Optic tract of both sides in section.

FIG. 6. Same as Fig. 3, but eye on right side is fairly large.

FIG. 7. Head is totally eyeless on left, where section goes through the inner ganglion, but misses the median. Right side has small eye.

FIG. 8. Frontal section of head totally eyeless on both sides.

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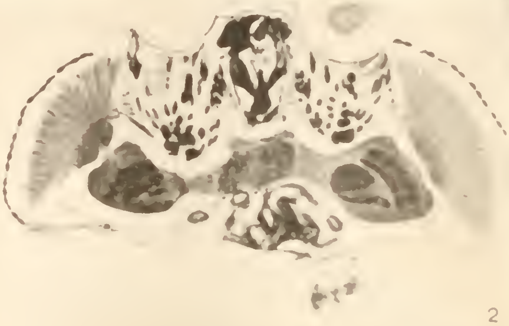

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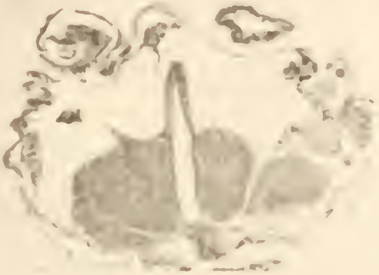

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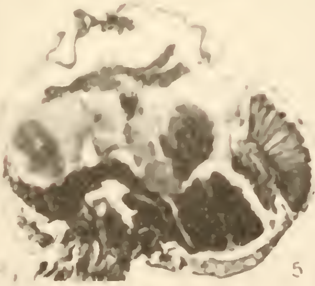

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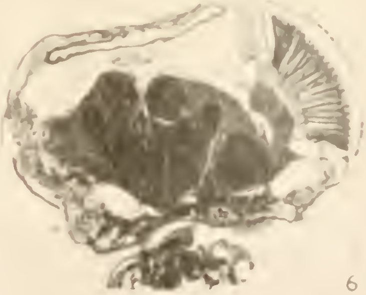

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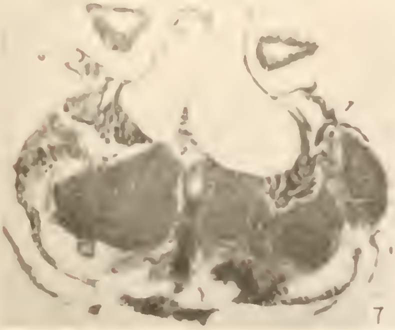

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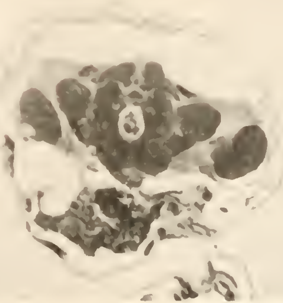

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RICHARDS AND FURROW.

A RECONNAISSANCE OF THE RELATION BETWEEN DESICCATION AND CARBON DIOXIDE PRODUCTION IN ANIMALS.

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Hull Zoölogical Laboratory, The University of Chicago and the Biological Laboratory of The University of Arizona.

I. Introduction.

A fairly comprehensive review of the literature on the desiccation of animals reveals work largely of two general types, (1) the effects of desiccation on the behavior of the animal as shown by Tower (1906), Hennings (1907), Jacobs (1909), Breitenbecker (1911), Shelford (1913, 1914a), Chenoweth (1917), Wesse (1917), Hamilton (1917), and Bodine (1923), and (2) the water content and the vital limits of desiccation of animals as determined by Leeuwenhoek (1701), Baker (1764), Doyére (1842), Semper (1881), Durig (1901), Babcock (1912), Schmidt (1918), Bodine (1921), and Hall (1922). From the standpoint of possible physiological changes resulting from water losses in animals, little has been done and it is the purpose of this paper to point out certain definite relations between desiccation and the physiological states of a limited number of species, as determined by the excretion of carbon dioxide.

The work has been made possible largely through the kind suggestions and helpful comments of Dr. W. C. Allee. Thanks are also due Dr. L. H. Hyman for assistance in the collection and identification of material.

II. Material.

Many animals were tried and rejected as unsuitable because of the inability to keep them quiet throughout the determinations. The material was selected so far as possible with regard to the natural habitat of the animals. The meal worm, larva of *Tenebrio molitor* Linn., a xerophilous animal, the larva of the eight-spotted forester moth, *Alypia octomaculata* Fabricius, a mesohygrophilous animal, and the slug, *Agriolimax campestris*

Binney, the slimy salamander, *Plethodon glutinosus* Green, the cricket frog, *Acris gryllus* Le Conte, all hygrophilous animals. The natural environments of these animals give a fairly wide range of maximum evaporation. With the exception of *Acris* and *Tenebrio*, the animals were kept in the laboratory only a few days and in all cases were under as nearly natural conditions as possible.

III. Methods of Desiccation and Carbon Dioxide Determination.

The desiccating apparatus consisted of a series of washing bottles so arranged that air could be drawn through sulphuric acid, granulated zinc, and glass wool into the desiccating chamber. The air from the desiccator was bubbled through a methyl orange solution for three hours to test for sulphuric acid. This test was repeated at intervals throughout the work and at no time were there indications of sulphuric acid in the air.

The respiratory apparatus devised by Osterhout was used to make the carbon dioxide determinations. Since this apparatus has already been described (Osterhout, 1918), a brief summary will suffice: the apparatus consists of a closed system in which air is circulated by means of a pump. There are two possible ways for the air to pass from the animal chamber, either directly into a Pyrex glass tube containing an indicator solution, or indirectly through a container of sodium hydroxide which removes the carbon dioxide from the air before it reaches the indicator solution. Thus, the desired color may be restored to the latter without the necessity of a change of indicator. This is highly advantageous because a series of determinations is possible without the admission of air to the closed system. In all experiments, 4 cc. of an aqueous solution of phenolsulfonephthalein were used. The indicator solution was made at the beginning of each experiment in sufficient quantity to last throughout one complete experiment, except when the experiment ran for several days, which occurred only in the work on the larvæ of *Tenebrio* and *Alypia*. A north skylight lamp and a white background made of absorbent cotton were employed to avoid the changeable color quality of daylight and furnish a constant light for work at night upon the critical comparisons of the known and unknown indicator solutions.

Wherever the respiration time of animals appears in this paper, it is to be taken as the time in seconds required by the animal, or animals, to excrete sufficient carbon dioxide to change 4 cc. of standard indicator solution from pH 7.8 to pH 7.2. No attempt was made to determine the actual quantities of carbon dioxide produced by the animals, since all comparisons were made on the basis of time required for the excretion of sufficient carbon dioxide to produce this standard change in pH value.

The control of the movements of the various animals in the respiratory chamber was on the basis of tropistic responses to light or contact. In no instance was cessation of activity produced through mechanical means, and no experiments have been recorded in this paper where the animal was moving when the carbon dioxide determinations were made. Movements could easily be detected through marked irregularities in respiration time.

Controls were run on all experiments. The control animals were kept under the same conditions as the experimental animals except they were not desiccated and exclusive of one control in the experiments on *Tenebrio*, neither controls nor experimental animals were fed during the experiment.

The animals were weighed in previously adjusted weighing bottles immediately after the carbon dioxide determinations were made. This proved preferable to weighing before the determinations were made since it reduced the stimulating effect of handling and consequently the animals came to rest in the respiratory apparatus more quickly. The animals undoubtedly lost weight in the animal chamber of the respiratory apparatus in some instances and gained weight in other instances, depending upon the state of desiccation, but the gain or loss inthe respiratory chamber was so slight compared with the loss in the desiccator that it was disregarded. All weights are given in milligrams as a matter of convenience.

The general procedure was the same for all of the experiments conducted. The normal respiration time of the experimental animal was determined; three consecutive readings were averaged for each determination. These readings varied somewhat because of the impossibility to detect always the exact end-points; in some instances the variation was as much as 2 per cent and throughout the work the variation was greater with the shorter respiration periods than with the longer. The animal was then weighed and placed immediately into the respiration chamber of the desiccator. The normal respiration time of the control was determined and its weight recorded while the experimental animal was being desiccated. This was repeated at intervals, usually until the experimental animal showed no signs of life. The intention was to obtain the rates of carbon dioxide excretion at approximate stages of from 5 to 10 per cent. loss in body weight. The time necessary for a loss in body weight of about 10 per cent. was previously determined with animals of similar weight.

IV. Experimental Results.[1]

A. Xerophilous Animals.

Meal Worm, Larva of Tenebrio molitor Linn.—These worms were kept in the laboratory in open Mason jars partially filled with air dried wheat bran. Five series of experiments were performed. Three worms were used in each experiment to reduce the respiration time. The controls of four of the five series were not fed. The controls of the fifth series were fed throughout the experiment. The first readings were the most difficult to obtain on account of the activity of the animals. A dark chamber was the only means used to keep the animals quiet, although it was found that animals placed with their bodies in contact with both the bottom and side of the animal chamber not only remained quiet but also were less inclined to aggregate. After 10 hours in the desiccator the meal worms were inactive when undisturbed. In the first four series, carbon dioxide determinations were made regularly at 24-hour intervals for six days. In the fifth series determinations were made at shorter intervals. Evaporation from the meal worm is very slow even in dry air. Hall (1922) found that it required 1,084 hours to desiccate a meal worm to 52.6 per cent. of its body weight. While this loss was a greater percentage of body weight than the average percentage of water contained in the body, the worm lived two days after desiccation.

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[1] Complete experimental data are on file in the University of Chicago Libraries.

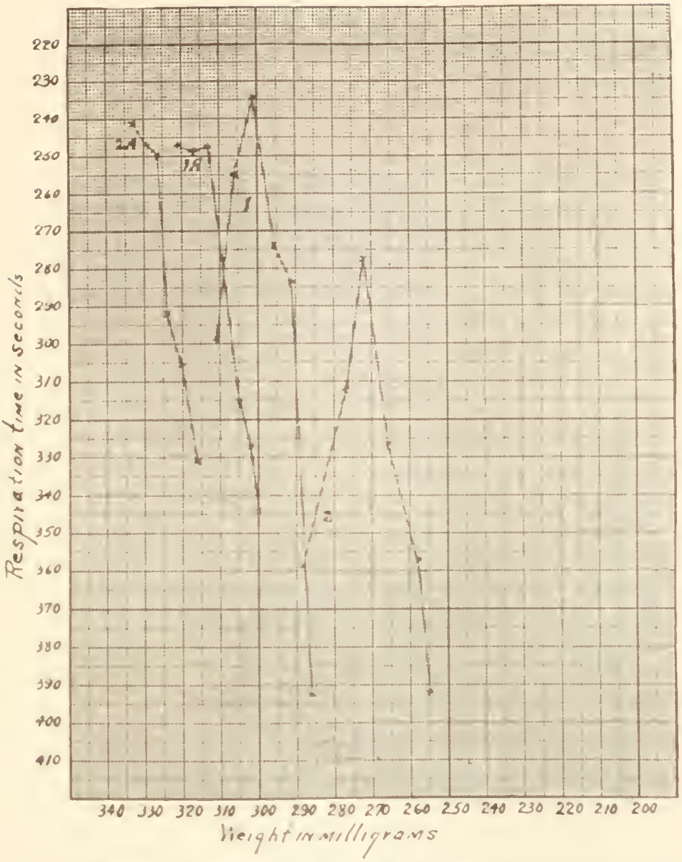

FIG. 1. The effect of desiccation on the carbon dioxide production of the meal worm. Points (x) denote determinations at 24-hour intervals. Curves 1A and 2A, controls of 1 and 2 respectively. Note marked decrease in carbon dioxide output at 72-hour determinations of both experimental animals and controls.

B. Mesohygrophilous Animals.

Larva of the Eight-spotted Forester Moth, Alypia octomaculata Fabricius.—This larva was taken from the Boston ivy (*Ampelopsis*), which grew on the walls of the laboratory, and placed directly into the animal chamber of the respiratory apparatus. Preliminary experiments proved the animal to be very active and stimulated by contact with the glass wall of the chamber. Fairly satisfactory results were obtained by placing a rough piece of wood, which had been oven dried and tested for carbon dioxide, on the bottom of the chamber upon which the animal became quiet when the chamber was darkened. This piece of wood was kept in the animal chamber throughout the experiment in order that the reduction in the volume of the apparatus would remain constant.

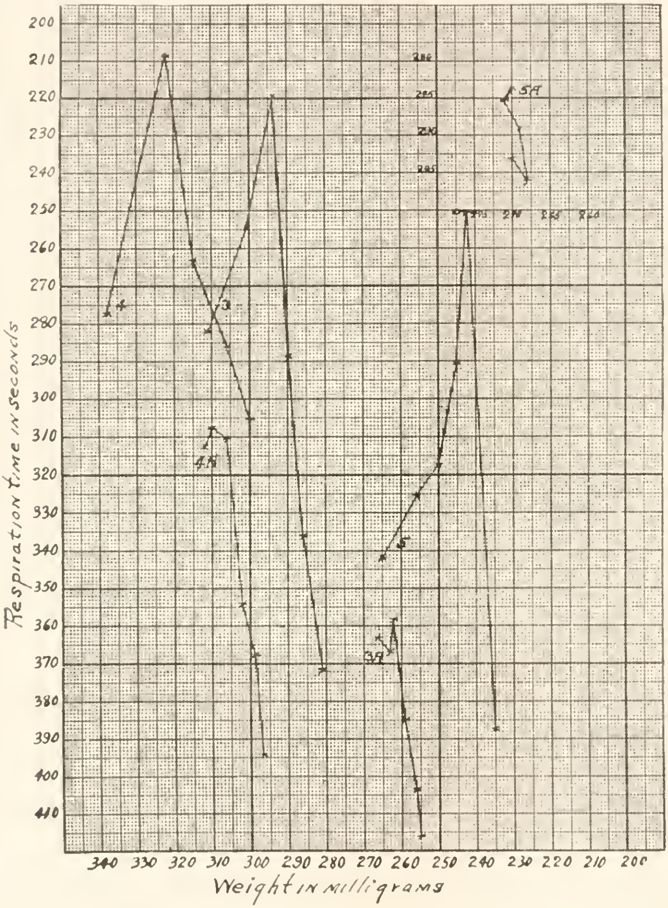

FIG. 2. The effect of desiccation on the carbon dioxide production of the meal worm. Experiments 3 and 4 with controls 3*A* and 4*A* on animals not fed during experiments and with determinations at 24-hour intervals. In experiment 5 the control animals, curve 5*A* (twice experimental scale) were fed. Point (*o–x*), curve 5, denotes carbon dioxide determination after 68 hours of desiccation.

Both the experimental animal and control lost about 55 per cent. of the body weight in 70 hours. The animals were still active but much reduced in size. The experimental animal and control showed a decrease in the rate of carbon dioxide production, the experimental animal from the first desiccation, and the control after 8 hours. The available data are insufficient to permit of any definite conclusions. However, two possibilities are suggested by this and several preliminary experiments: one, the partial closure of the spiracles to retard the loss of water, and the other, the effects of starvation. Similar results were obtained in trial experiments with the larvæ of two other species of Lepidoptera (unidentified) and the centipede, *Scolopendra heros*.

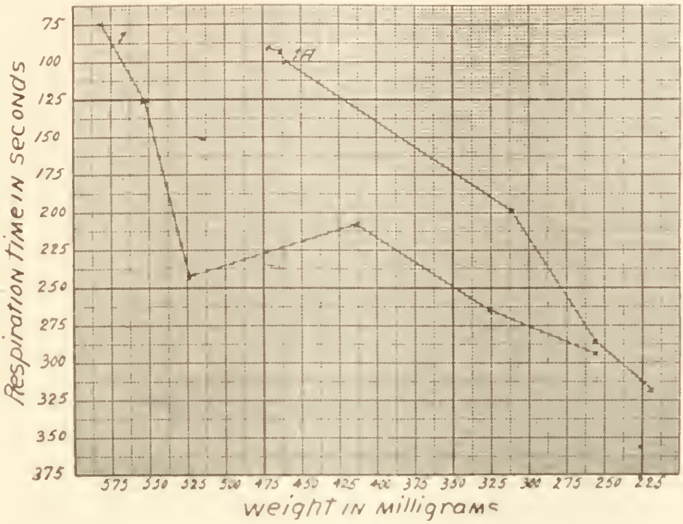

FIG. 3. The effect of desiccation on the carbon dioxide production of the larva of the eight-spotted forester moth. The time intervals of determinations (*x*) were the same for the experiment, curve 1, and control, curve 1*A*; first, 3 hours, second, 5 hours, third 24 hours, fourth, 18 hours and fifth 14 hours.

C. Hygrophilous Animals.

Slug, Agriolimax campestris Binney.—The experimental animals were taken from their normal environment in a greenhouse and placed in the animal chamber of the respiratory apparatus. The control animals were kept in moist filter paper in a closed dark chamber where the rate of evaporation and temperature were approximately that of the normal environment. Single individuals were used in each of the four series of experiments recorded. The animal chamber of the respiratory apparatus was covered with black paper and a small opening at one side permitted of observation without disturbing the animal by the movements involved in the manipulation of the apparatus. The slugs usually came to rest in a short time in the darker region of the chamber. Series of determinations were made until the readings became constant.

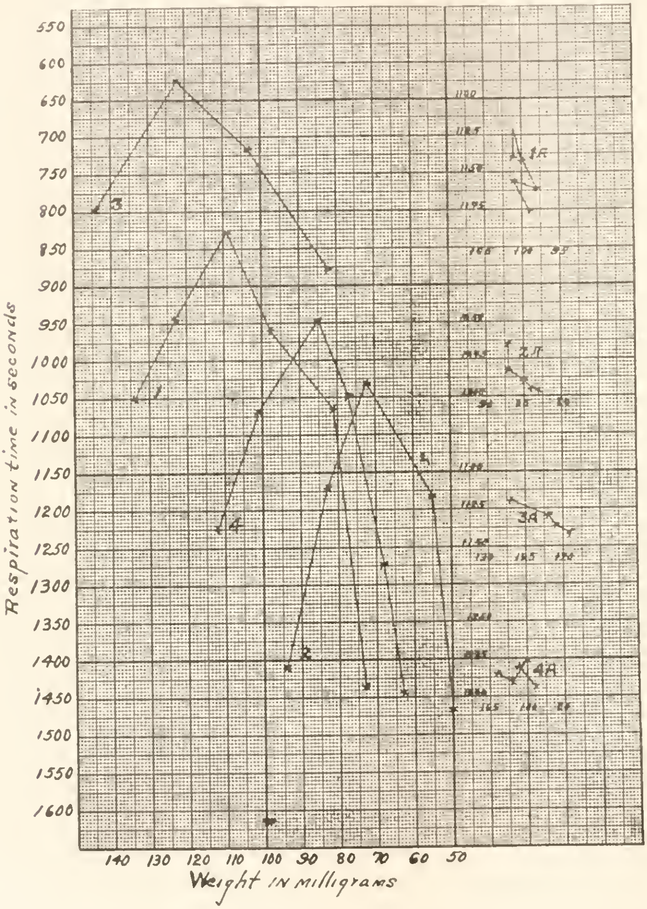

FIG. 4. The effect of desiccation on the carbon dioxide production of the slug. Control curves indicated by letter (A) and enlarged to twice the scale of experimental curves. Total time of each experiment about 8 hours.

The rate of carbon dioxide production increased with desiccation until there was a loss of water equivalent to from 20 to 30 per cent of the body weight when the rate fell below normal and continued to decrease with further loss of water. The time between the first and last determinations was too short to permit the starvation factor to enter into the results. The controls lost some water in the duration of a series, a period of about 8 hours. One control lost 6.29 per cent. of the body weight, the greatest loss of any control, while the experimental animal of the same series lost 43.05 per cent. of the body weight. The respiration rates of the controls seemed to be little, if at all, affected by the slight losses of water.

Salamander, Plethodon glutinosus Green.—The salamanders gave up water very quickly. Results show that 10 per cent. of their body weight was lost in 15 minutes of desiccation. The greatest loss of the four individuals experimented upon was 42.92 per cent. of the body weight, distributed over a period of 8 hours.

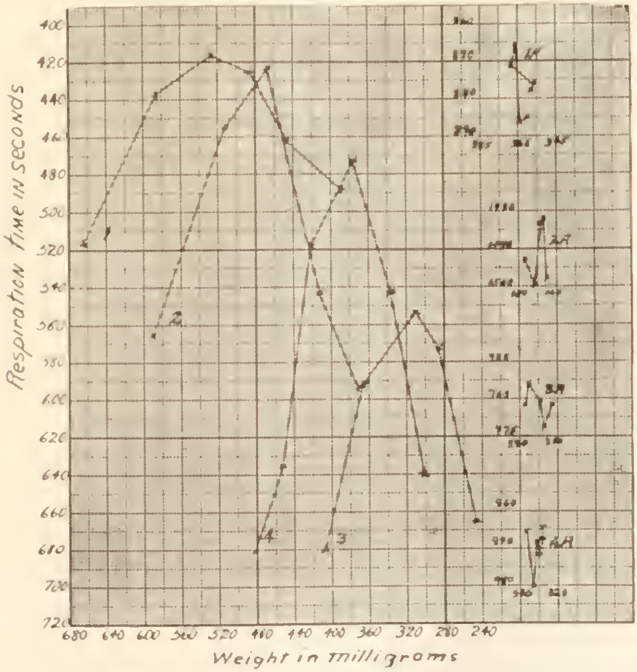

FIG. 5. The effect of desiccation on the carbon dioxide production of the salamander. Control curves indicated by letter (A); numbers correspond to the experimental curves. Control curves twice experimental scale.

This individual lived 6 hours after the last desiccation. Hall (1922) desiccated *Ambystoma punctatum* to 47 per cent. of their body weight without loss of vitality. The salamanders proved easy to handle, oriented themselves negatively to light in the darker region of the animal chamber and remained quiet. When placed in the desiccator for the first time, the animals were quite active for 5 minutes, never longer. Carbon dioxide determinations prior to losses of 20 to 25 per cent. in body weight showed a rapid increase while continued desiccation resulted in a decrease in carbon dioxide output. The control animals lost very little water, not enough to appreciably affect the rate of carbon dioxide production.

Frog, Acris gryllus Le Conte.—Adult frogs of this species which

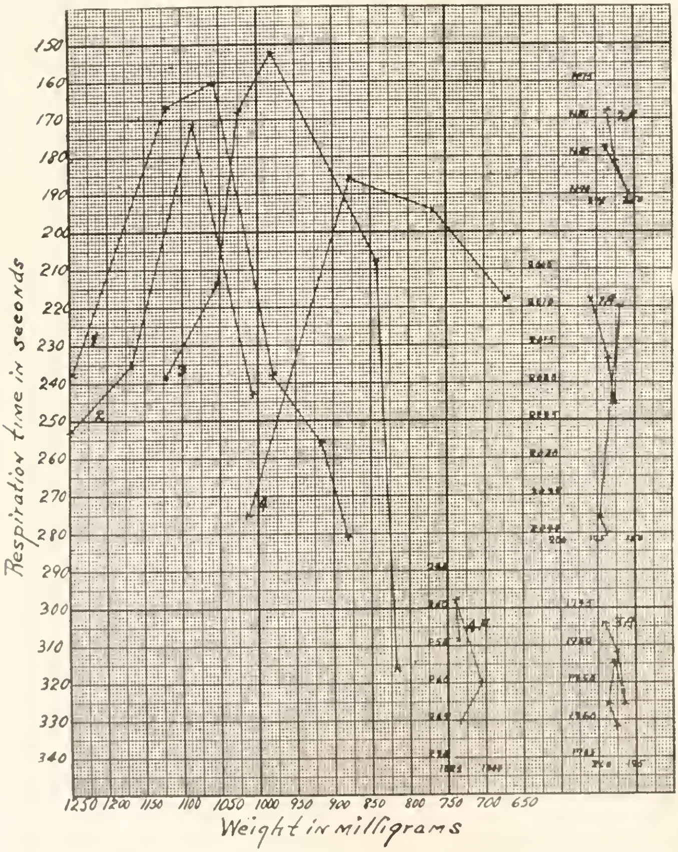

FIG. 6. The effect of desiccation on the carbon dioxide production of the frog. Points (*x*) on control curves (indicated by letter *A*) correspond to determinations on experimental curves. Control curves twice experimental scale.

had been kept in the laboratory for several days were used. To quiet the animals proved rather difficult. A dark chamber with a small opening in the paper covering caused the animals to orient themselves towards the light. After being handled the respiratory rates of the animals returned to normal very slowly, that is, a number of consecutive readings showed a decrease in rate before they became constant. This suggested the possibility of oxygen depletion within the closed system. Krogh (1916) in summarizing the work of several authors concluded: "In cold blooded animals oxygen consumption is practically independent of the oxygen pressure down to about 2 per cent. pressure." Fresh air was admitted to the animal chambers without disturbing the animal, by the removal of the indicator tube. This had no effect upon the rate of carbon dioxide production, so it was assumed that the animal had obtained sufficient oxygen. The rate of carbon dioxide production in the desiccated frogs of the four series of experiments increased with loss in weight until the loss amounted to from 15 to 20 per cent. of their body weight. The rate diminished subsequently to the completion of the experiment. The total time of a single experiment was less than 14 hours and hardly permitted of starvation as a factor in the decrease in respiration rate. The control animals lost very little water, from 0.26 to 1.47 per cent. The respiratory rates of the control frogs were fairly constant throughout the work.

V. Discussion.

These investigations show that desiccation stimulates the physiological activity of certain animals and that the increase in irritability is followed by marked depression. While there is no literature on the effects of desiccation on the carbon dioxide output of animals, it is interesting to note that Bodine (1922) obtained somewhat similar results from the action of ether, xylol and acetone upon grasshoppers, and concluded that anesthetics have physiological effects other than on respiration. Shelford (1913), with behavior methods, found that animals subjected to air of low humidity were more active than normal ones, and if kept under such conditions, the period of activity was followed by a period of depression, and suggested that the increased irritability was probably due to the concentration of the blood and tissue fluids. Since the present results are based on physiological determinations, they are hardly comparable with results from behavior studies, however, it seems reasonable to suppose that if this were the entire cause of the stimulation, the increased irritability would continue, with additional losses of water, to a point nearer the vital limit of desiccation than has been found in the present experiments. Also, Shelford refers to irritability as evidenced by the activity of the animals, while these experiments show that the increased metabolic activity continues long after the animals become inactive. To cite a special case, the meal worms were active for the first 6 to 10 hours in the desiccator but the respiratory rate increased for more than 60 hours.

Since meal worms with a water loss of less than 1 per cent. of the body weight gave an increase in respiratory rate of more than 10 per cent., increased concentration of body fluids could hardly be the principal factor in causing the increase and suggests that this is, in part, a function of the nervous system. The truth of this assumption is open to investigation and further work will be done along this line.

There is a rough correlation between the highest respiratory rates and the vital limits of desiccation. According to Hall (1922), the vital limit of the frog is 41 per cent. and that of the salamander 47 per cent. The highest respiratory rate of the frog, from an average of four experiments, corresponded to 13.66 per cent. loss of body weight and with the salamander to 21.87 per cent. loss of body weight. The xerophilous animals gave up water very slowly and in proportion to water losses were stimulated more than the hygrophilous animals.

The decrease in carbon dioxide elimination with further desiccation is even more difficult to explain. No literature on studies pertaining to either starvation or desiccation of the larvæ of *Tenebrio* and *Alypia* has been found, but Hill (1911) showed that the heat production of the frog decreased with starvation and Child (1919), Hyman (1919) and Allen (1919) who worked with *Planaria*, and Bodine (1921) with grasshoppers, all pointed out that starvation decreased the rate of carbon dioxide production. The results from the meal worm experiments indicate a stimulation from desiccation until the depression of food starvation became a greater factor. Particularly, since the respiratory rates of both the experimental animals and controls decreased after 48 hours of desiccation without food, while the fed controls showed only slight variations. Water starvation undoubtedly increases the depression of food starvation, but this fails to explain the decrease in respiratory rates of animals not affected by starvation.

In work on pithed frogs, the author has observed that handling often produced reflex movements, but after the frogs had lost considerable water these reflex movements did not occur, and furthermore, were not induced when the sides of the body or feet were pinched. This suggests the possibility that after the nerves have lost a certain percentage of water, the passage of the nervous impulse is retarded or inhibited. If this be true, in part or whole, the highest respiratory rate should immediately precede the disappearance of reflex movements, but no data are available on this point. Durig (1901) found that the nerves of a frog transmitted more slowly and the latent period of the muscles increased with water losses from 9 to 30 per cent. of the body weight, so that the decrease in respiratory rate is what might be expected if this were true.

Until more data are available, it is difficult to suggest the causal factors, but the effect will probably be found to be in part caused by accumulation of waste products due to poor elimination and also to inadequate distribution of food and oxygen to the cells. It is also highly probable that the decrease in water content has a direct action in slowing down certain of the oxidative processes. Decreased metabolism from any or all of these causes would appear in the experiments as conducted.

VI. SUMMARY.

1. Desiccation is followed by marked changes in the physiological states of certain animals.

2. *Agriolimax campestris* Binney, *Plethodon glutinosus* Green, *Acris gryllus* Le Conte, and larvæ of *Tenebrio molitor* Linn show a definite, positive, correlation between desiccation and carbon dioxide production.

3. The above-named animals show a gradual increase in carbon dioxide production when desiccated to less than one half of the possible vital limit. Continued desiccation is followed by a

decrease in carbon dioxide production, which falls below normal before the vital limit is reached.

4. The larvæ of *Alypia octomaculata* Fabricius show a decrease in carbon dioxide production in all stages of desiccation.

5. Xerophilous animals give up water more slowly and in proportion to water losses are more stimulated by dry air than hygrophilous animals.

6. Water starvation increases the depression of metabolic activity caused by food starvation.

7. Extremely dry air is a protoplasmic irritant.

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EXTERNAL PARASITES OF BIRDS AND THE FAUNA OF BIRDS' NESTS.[1]

IRENE D. DOBROSCKY.

There have been comparatively few papers published on the parasites of birds. The work of the writer on this subject was carried on at Cornell University with material collected in the vicinity of Ithaca, New York.

The external parasites of the birds examined were all Arthropods falling into three orders,—Diptera, Mallophaga, and Siphonaptera. The dipterous parasites belong to the genus *Protocalliphora* of the Family Calliphoridæ. The majority of Calliphorine flies are scavengers that live on decaying animal and vegetable matter. The genus *Protocalliphora*, however, is a small group of flies parasitic on young birds. The larvæ are intermittent blood-sucking obligate parasites. There is some controversy as to whether they are confirmed ectoparasites or not. Bezzi ('22) states that the subcutaneous tumors caused by fly-larvæ are in many cases erroneously attributed to the *Protocalliphora* and that other Calliphorine flies may be the agents. Since the *Protocalliphora* are intermittent feeders it is impossible for them to become permanent ecto parasites.

The nomeclature of this group is somewhat involved. In earlier literature the genus *Protocalliphora* is referred to under the names of *Phormia* and *Protophormia*. *Avihospita* is a later synonym. There are at least five *valid* world species in this genus. In Europe there are two distinct species—*P. azurea* Fallen and *P. cærulea* Robineau-Desvoidy. According to the studies of the writer based on larval and pupal forms, and on adults by R. C. Shannon, there are at least three distinct species in North America. These are *P. splendida* Macquart, *P. avium*, and *P. hirudo* (a Western form), the latter two described by Shannon and Dobroscky ('24).

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[1] The writer wishes to express her appreciation for help, suggestions, cooperation and determinations to Dr. R. Matheson, Dr. O. A. Johannsen, Dr. J. Bequaert, Dr. W. T. M. Forbes, Professor G. W. Herrick, C. R. Crosby, R. C. Shannon, J. W. Folsom, F. C. Fletcher, H. Good, C. H. Curran, L. S. West, J. R. Mallock, H. C. Huckett, and to the numerous persons who aided in collecting the birds' nests.

The writer's work upon parasitic larvæ has been confined to *P. avium*. This larva, which is a parasite of the crow, is amply described and figured by Coutant ('15) under the name of *P. azurea*. The larval period is from ten to thirteen days, and consists of three stages. Larvæ measuring from 2 mm. to 15 mm. were found in the nests of crows. If a third stage larva has not attained full size, and cannot obtain food, it will pupate, and the emerging adult will be smaller than usual.

Pupal cases belonging to *P. avium* and *P. splendida* collected from the nests of several species of birds were carefully studied. Within each species differences in the pupal cases of the parasites from different bird hosts were observed. Since the adult flies also show distinct differences, according to the host, it seems probable that the variations are due to heredity, with high specificity, and are not due to the fact that the larvæ have fed upon different hosts. The most noticeable variation in the pupal cases was in the size and arrangement of spines around the stigmal area. The spines in the parasite on the house wrens were .05 mm. long, while in the puparia found in the nest of the scarlet tanager they were so minute that they seemed like mere stipples. In some the spines are inserted directly into the underlying chitin and in others they are on raised ridges. A more detailed description of these host to host variations, with illustrations, is given in the writer's thesis on this subject on file at Cornell University.

The method of hibernation of this parasite is not known. It seems most probable that it winters over as an adult. Since the eggs must be laid in new nests in order to hatch in the immediate presence of young nestlings, the egg stage is ruled out as a method of hibernation. Of the hundreds of pupal cases collected during the fall, winter, and spring months none were alive. They were either empty, or dead and dried up, or parasitized by a small hymenopteron, of which note will be made later.

The bird hosts of the North American species of the genus *Protocalliphora* are as follows:

I. Those observed by the writer: Robin, bluebird, house wren, crow, scarlet tanager, indigo bunting, chipping sparrow, song sparrow, catbird.

II. In addition those listed in the joint paper by R. C. Shannon and the writer based on material in the U. S. National Museum: Brown thrasher, cardinal, cooper's hawk, long-eared owl, cliff swallow, western robin, western horned-lark.

III. In addition those listed from California by O. E. Plath: Yellow warbler, green-backed goldfinch, willow goldfinch, Nuttall sparrow, California purple finch, California linnet, California brown towhee, rusty song sparrow, Oregon towhee, russet-backed thrush, cedar waxwing.

IV. In addition those recorded by Bezzi: Bank swallow, swamp sparrow, white-throated sparrow, white-crowned sparrow.

V. Henshaw ('08) records: Woodpeckers.

Since these parasites are so prevalent, no doubt in the course of further investigation, many more species of birds will be found to be parasitized.

ECONOMIC IMPORTANCE.

The results of the writer's collecting show the following amount of infestation:

Host.	Nests Examined.	Nests Infested.	Deaths.
Robin	202	28	None.
Crow	17	7	None.
Bluebird	5	5	Three.
House wren	26	9	Two.
Indigo bunting	2	1	None.
Scarlet tanager	1	1	None.
Catbird	15	1	None.
Chipping sparrow	31	1	None.
Song sparrow	20	1	None.

The number of pupæ in a nest varied. In robins' nests there were from 1 to 20 pupæ; from 10 to 50 were found in the bird-houses of house wrens and bluebirds. Other nests, except those of the crow, contained usually from 1 to 4 pupæ. The crows' nests however, contained anywhere from 20 to 343. The nest in which the 343 larvæ of all sizes, were found, harbored three young crows. These were much smaller and weaker than crows of their age usually are.

A parasite with such a wide range of valuable birds as hosts must cause considerable harm. The problem, therefore, presents a serious economic aspect. Henshaw ('08) states that this parasite on bluebirds produces almost 100 per cent. mortality. Plath in California found a mortality among nestling birds of 5 per cent. to 10 per cent. Coutant and Dufour report no mortality among crows and swallows. The experiments of the writer indicated no mortality, but young crows from parasitized nests showed a marked retardation of growth, and young robins became so weakened that a slight exposure to cold was sufficient to cause their death. However, in collecting, the parasitized nests of bluebirds and house wrens were found to contain the bones of nestlings. The presence of the parasites seems to indicate that they were responsible for the deaths. No doubt in the natural habitats the presence of these parasites weakens the young birds and indirectly causes their death by lowering their resistance to unfavorable conditions and causing them to fall an easy prey to other animals.

Mallophaga, or bird-lice, are parasites which have accompanied the whole phyletic history of birds. Harrison ('14) who has written a very interesting paper on this subject says, "I would suggest that the adoption of a parasitic habit by mallophagous insects occurred even as far back as the late Mesozoic time." In their primitive form he says they parasitized both birds and marsupials before the true mammals differentiated out. H. E. Ewing identified some bird lice collected by the writer from a crow as *Myrsidea subæqualis* Lay.

One does not usually think of fleas as parasites on birds, but they are to be found quite frequently in bird nests. Numerous fleas were collected from the nests of bluebirds, house wrens, bank swallows, and house sparrows. Adult fleas, in copulation, were taken from the nests of bluebirds, house wrens, and house sparrows which were collected in the month of January. A bank swallow's nest examined in June contained enormous numbers of larvæ. Posted at the lower part of the hole which is the entrance to the inhabited nests, are the adult fleas. They probably attach themselves to the parent birds and are so spread throughout the colony. All these fleas noted above were determined by R. C. Shannon as belonging to the genus *Ceratophyllus*.

THE FAUNA OF BIRDS' NESTS.

Besides the inhabitants that parasitize birds, there were found a great variety of other insects and animals of lower orders. They use the nests as a place to hibernate or to breed; in the latter case the larvæ feed on the animal and vegetable debris in the bottom of the nest. These commensals are namely sow-bugs, spiders and mites, and numerous species of insects.

The common sow-bug was encountered several times in the nests of robins. As these nests are usually damp it is not surprising that this animal should be found in them.

Spiders of several species are found in almost all bird nests, especially during the winter months. The majority of the spiders found in the nests of robins, crows, house wrens, bluebirds; chipping, song, and field sparrows, catbirds, and woodthrushes were in the immature stages. Egg-sacs were also found in the nests of bluebirds and house wrens during the winter months.

Numerous mites were found in the nests of crows, robins, house wrens and house sparrows. These were identified by H. E. Ewing as belonging to five distinct species.

Of the Collembola or spring-tails two species were encountered frequently in the nests of crows, woodthrushes, catbirds, song sparrows, red-eyed vireos, and robins. These were identified by J. W. Folsom as *Entomobrya assuata*, a new species, yellowish orange in color; and a smaller purple species, *Isotoma arborea.*

A species of Psocids, a group to which the common book-louse belongs, was found in nests of robins and house sparrows in appreciable numbers all year round.

Two species of thrips (Physopoda) occured in the nests of robins and field sparrows. *Limothrips denticornis* is an European species three records of which have been reported from New York. The writer collected this species on two occasions from bird nests and there is another record from wheat heads. The common mullein thrips, *Neoheegeria verbasci*, was found in a number of nests.

Several adult Hemiptera, or what may be truly termed "bugs," were found in the nests of robins. Where they were probably hibernating. They were leaf-eaters belonging to the Family Tingitidæ and the Family Capsidæ.

Homoptera, represented by the pear psylla and leaf-hoppers, were found in robins', catbirds', and song sparrows' nests. They were adults and were using the nests for hibernation purposes.

It is not at all uncommon to find moths in connection with bird nests. The Tineids, to which our common clothes moth belongs, are found in nests of the robin, crow, house sparrow, redstart, and chipping sparrow. Larvæ pass the winter in pupal cases made of the grasses of the nest and resemble caddis-fly cases. Adults emerge in May when there are fresh nests in which they lay their eggs, according to the statement of one European worker. The adults were identified by W. T. M. Forbes as *Tinea carnariella*. Several adults of *Carnarsia ulmiarrosorella* emerged from robins' nests in the early part of June. As the larvæ feed on shade trees, they probably use the nest as a place to hibernate.

Of Diptera or flies there were more species found than of any other order of insects. Several of them pass through their complete life cycle in the nests.

Calliope flaviceps, a small fly belonging to the Family Sapromyzidæ, is encountered frequently and in large numbers in the nests of the robin, woodthrush, redstart, song sparrow, goldfinch, catbird, red-eyed vireo and chipping sparrow. Numerous small white larvæ and flat yellow pupæ are found in the bottom of nests in the fall, winter, and spring months. The larvæ feed exclusively on the debris and seeds they find in the nests. Adults emerge early in May. It would seem that the nests of birds are the natural habitat of this species but the fact has not been noted before.

Four species of Anthomyidæ were found. Larvæ and pupæ of *Anthomyia pluvialis* were found in nests of the robin, bluebird and chipping sparrow. Adults emerged from March to May. Larvæ of *Fannia canicularis* were found in the nests of robins, bluebirds and house sparrows. Adults of this species emerged in May. *Hydrotœa nidicola* is a new (manuscript) species described by J. R. Malloch. Pupæ were found in robins' nests, and adults emerged in the latter part of April. *Hylemyia spizella* is also a new species to be published by H. C. Huckett. The flies emerged from robin and chipping sparrow nests in March.

Clausicella usitata is a small Tachinid found in the nests of robins, crows, bluebirds, and song sparrows. As all the Tachinidæ are parasitic on other insects, this species was probably parasitizing the other Arthropods in the nests.

Three species of Sarcophagids or flesh-flies were found. *Sarcophaga sinuata* was found in the nests of the catbird, and field sparrow. Adults emerged during the month of March. *Sarcophaga spuria* and *Lacchoprosopa avium* are both new (manuscript) species described by C. H. Curran. The former was found as a pupa, and the adult emerged under laboratory conditions in March; the latter species, obtained from a crow's nest, emerged in March, also the larvæ of Sarcophagids live on decaying vegetable matter and animal refuse, and therefore, birds' nests are ideal places for them.

Small adult flies of the Oscinidæ, Phoridæ, and the Mycetophilidæ were found in crows' nests in the spring months.

Long thin dipterous larvæ with nineteen segments were found in house sparrows' nests. These were reared and proved to be *Scenopinus fenestralis* the so called window-fly. The larvæ feed on the straw and twigs of the nests.

Numerous species of beetles were found in nests. The majority were in the adult stage and seemed to be using the nests for hibernation purposes. However, in the house sparrows' nests numerous larvæ of Dermestids and Tenebrionids were found, which feed on the straw of the nests. A larva of *Malachius æneus*, an European beetle, was found in a robin's nest and reared to an adult. Some of the adult beetles found in robins' nests were: *Ptinus fur*, *Crioceris asparagi*, *Prasocuris vittata;* and Curculionid, Scymnus, Melanophthalma and Bembionid species.

The Hymenoptera found were either parasites of other insects or used the nests for hibernation. One small green hymenopteron, identified as *Mormoniella brevicornis*, was found frequently in nests which contained pupæ of *Protocalliphora*, the bird parasite referred to in this paper. These small hymenopterons parasitize the muscid pupæ, and about fifteen issue from one pupal case. Other Hymenoptera found in nests were identified as *Megastigmus nigrovariegatus*, *Passalæcus* species, *Eucoila* species, *Hyperacmus tineæ*, *Aenoplex betulæcola*, *Apanteles carpatus*, and *Tetrastichus* species. These were found as adults which were probably hibernating during the winter months.

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THE METHOD BY WHICH *TRICHONYMPHA CAMPANULA*, A PROTOZOÖN IN THE INTESTINE OF TERMITES, INGESTS SOLID PARTICLES OF WOOD FOR FOOD.

L. R. CLEVELAND.[1]

The intestinal protozoa of wood-feeding termites represent a unique and peculiarly interesting faunal association. The protozoa, very abundant both in form and number and completely filling the large and much distended gut of their host, take into their bodies and digest practically all the wood which the termites eat. The termites themselves cannot digest wood or cellulose, and hence cannot live on wood, their normal diet, without protozoa to digest it for them.

From one third to one half the body of *Trichonympha campanula*, the largest and principal wood-ingesting protozoön harbored by *Termopsis*, is filled with wood fragments, though the method employed in taking them in has been a complete mystery.

It is possible that many of the termite protozoa take in wood particles from the intestine of their host similarly to *Trichonympha campanula*. Most of them, like *T. campanula*, have no cytostomes.

Several suggestions have been made as to the possible methods of food ingestion by these protozoa. Leidy ('81), in his account of termite flagellates, called attention to the presence of food bodies and the lack of any visible channel for their entrance into the body. Kent ('84) claims to have discovered an oral aperture at one side of the body, a short distance from the anterior end. From this opening he traced a narrow œsophagus emptying into a digestive cavity in the posterior region of the body. He studied the organisms in thinly diluted milk, and says that both the pharynx and digestive tract were frequently filled with milk corpuscles. The organism which Kent observed was taken from an unknown termite of Tashmania and belonged, according to

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Kent, to the genus *Trichonympha* (*T. leidyi*), although Koidzumi ('21) has questioned (and perhaps rightly so) whether it belonged to this or another closely related genus.

Porter ('97), working with *Trichonympha agilis* from *Reticulitermes flavipes*, attempted to confirm Kent's work, both in the living animal and by means of sections of the body, but was unable to find any trace of an oral aperture. He suggests that the food particles may be drawn to the posterior part of the body by the flagella and there ingested through the thin pellicle. Concerning the possibility of the wood particles being ingested at the anterior end, Porter says: "It seems highly improbable—to say nothing of the absence of a permanent oral aperture—that solid food should pass through this region so quickly that not a single case of its presence in this part should have been discovered by any of those who have studied these parasites."

Of Porter's suggestion, Kofoid and Swezy ('19) remark: "Unfortunately the evidences for this are unconvincing." It is the opinion of these authors that in *Trichonympha campanula* the centroblepharoplast may function as a cytopharynx; yet they say, "that the centrosome should form part of the mouth structures, however, seems hardly plausible, but scarcely less so that its food should be taken in at the posterior end of the body." Cutler ('21) says, "a grave objection to this conclusion [the conclusion of Kofoid and Swezy, '19] is that food particles are never found in the anterior end of the body. One is thus drawn to the belief that food is incorporated into the body at the posterior region, though the method is still unknown."

More recently Swezy ('23) has described a pseudopodial method of food ingestion in *Leidyopsis* and *Trichonympha*. But it seems to me what she has really seen and has described is only the last stage of the unusual ingestion of very large pieces of wood, by the method described in this paper. Certainly, not one individual in ten thousand is ever seen to take in such large pieces of wood as she has figured. Instead, they feed on the smaller pieces of wood which they may ingest much more readily and which do not distend their bodies in all kinds of abnormal shapes, such as Swezy figures to be the method of food ingestion.

Perhaps the chief reason why Swezy did not see more of the process is due to the fact that she did not find a suitable medium in which to observe the organisms while feeding. She only observed them in the intestinal fluid and in this, as anyone who has ever studied termite protozoa knows, the number of protozoa present is far too great to permit almost any kind of an observation whatever, and, in addition to this difficulty, the protozoa immediately become decidedly abnormal and die very quickly. The best Swezy could do was keep the protozoa alive for six hours, so she states. This investigator also used physiological salt solution to dilute the intestinal contents and states that "the flagellate tends to round up immediately, becoming greatly distended and dissolving a few minutes after being placed on the slide." The osmotic pressure of such a solution is much too high for *Trichonympha* but not for *Trichomonas* from the same host. Half this amount of salt is far better.[1] But better still is:

NaCl	0.3	grams
$CaCl_2$	0.02	"
KCl	0.02	"
$MgCl_2$	0.01	"
NaH_2PO_4	0.001	"
$NaHCO_3$	0.01	"
H_2O	100 cc.	
Löffler's blood serum	0.5	grams.

This is filtered and used immediately. Instead of the serum, 5–10 grams of fresh *Termopsis* feces may be thoroughly shaken up in the above mixture, and may be used with or without filtering. If filtered, a small amount (1–2 grams) of finely powdered lignocellulose is added. In this way, many *Trichonympha* live ten days and longer and may be observed as constantly as desired, under a sealed cover glass or otherwise.

Method of Food Ingestion.

When the flagellate takes in food, the posterior end, owing to contraction of the longitudinal endoplasmic myonemes, becomes invaginated, thus forming a cavity or temporary cytostome (Fig. 6). These myonemes, about two micra in breadth and easily observed in living material, are the principal organelles concerned with body movement. They begin very near the anterior tip (somewhere near, possibly within the centroblepharoplast) and run almost parallel, widening out slightly posteriorly, for approximately three fourths the body length (Fig. *A*). Posterior to their termination, there is a hyaline, much less differentiated region, void of fibers, flagella and myonemes. This region flows backwards and forwards (Fig. 8) with much freedom of movement when compared to the rest of the body. It, in fact, sometimes extends posteriorly more than twice the distance shown in Fig. 8. At times great masses of wood particles are seen adhering to the outer (and probably sticky) surface of the body in this region. They enter the body when the posterior end invaginates.

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[1] More recent observations show that 0.2 per cent. NaCl is the best salt solution for observing the protozoa of certain tropical termites.

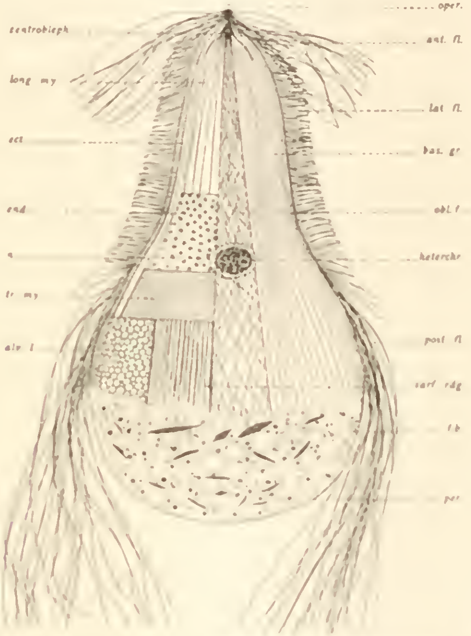

FIG. *A*. Diagrammatic figure of *Trichonympha campanula*. Sections of the body show the structures found at different levels. Surface ridges form the outer layer with their rows of flagella; beneath are successively the oblique fibers, alveolar layer and transverse myonemes. In the endoplasm are the longitudinal myonemes.

Abbreviations: *alv. l.*, alveolar layer; *ant. fl.*, anterior zone of flagella; *bas. gr.*, basal granules; *centrobleph.*, centroblepharoplast; *ect.*, ectoplasm; *end.*, endoplasm; *f.b.*, food bodies; *heterch.*, heterochromosome; *lat. fl.*, lateral zone of flagella; *long. my.*, longitudinal myonemes; *n.*, nucleus; *obl. f.*, oblique fibers; *oper.*, operculum; *per.*, periplast; *post. fl.*, posterior zone of flagella; *surf. rdg.*, surface ridges; *tr. my.*, transverse myonemes. × 300. (Figure and legend after Kofoid and Swezy.)

The Successive Stages in Food Ingestion.—A flagellate just previous to taking in food (Fig. 1) usually has few wood particles inside the body; whereas at other times about one third (the posterior third) of the body is always filled with the wood particles. First the longitudinal myonemes contract, thus causing the posterior end to become more rounded and slightly flattened centrally (Fig. 2). It is quite possible that the transverse myonemes expand at the time, though it has been impossible to actually observe this. In the next stage (Fig. 3) the body is more contracted and hence shorter; this contraction, owing to the fact that the inner or central portion was pulled forward while the outer and more rigid ectoplasmic portion did not invaginate, results in the formation of a cup-shaped cavity (Fig. 5) lined with the wood particles which adhered to the outer and undifferentiated region prior to invagination. Now the cytoplasm of the most posterior portion of the undifferentiated region soon begins to flow backward, thus narrowing the posterior end of the cavity (Fig. 6). This continues until finally the cavity is completely closed, the wood particles remaining inside (Fig. 7).

Other *Trichonympha* sometime get caught in the cavity just as it begins to close (Fig. 9), and may be seen swimming in this position for ten minutes or more, for caught individuals free themselves with great difficulty. This, of course, is not a common occurrence, though five such observations were made within four hours. In three termites, out of more than 500 that were examined, perhaps half the *Trichonympha* present had other *Trichonympha* partially ingested and securely fastened inside their bodies. The cause of this peculiar phenomenon is entirely unknown. Living individuals of *Trichomonas termopsidis*, a much smaller flagellate always associated with *Trichonympha*, are sometimes ingested along with wood particles, though not in sufficient quantity perhaps to be of much food value. *Leidyopsis sphærica*, another protozoön closely related to and associated with *Trichonympha* in *Termopsis angusticollis*, takes in wood particles similarly to the method described here. It has also been observed killed and half ingested by *Trichonympha*. This, however, is a rare observation.

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EXPLANATION OF PLATE I.

FIGS. 1–7. Method of wood ingestion in *Trichonympha campanula*. All figures × 180.

FIG. 1. Before the ingestion of food (wood). Note the particles of wood adhering to the posterior surface of the body.

FIG. 2. First stage in the process of ingestion. The longitudinal myonemes (see text Fig. *A*) have contracted and the posterior end has thus become flattened with the wood particles sticking to the outer and probably sticky surface of the body.

FIG. 3. By greater contraction of the longitudinal myonemes a cup-shaped cavity is being formed by an invagination of the posterior end.

FIG. 4. Cup-shaped cavity or temporary cytostome completely formed.

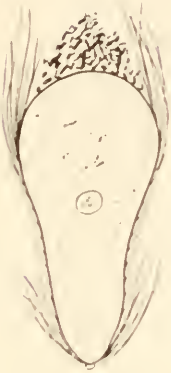
FIG. 5. The cavity is closing by a flowing together or backward of the clear, hyaline and undifferentiated portion of the body. Protoplasm in this region is free to flow back and forth for a considerable distance. When very large pieces of wood are ingested, it flows back around them pseudopodia-like; but this is unusual.

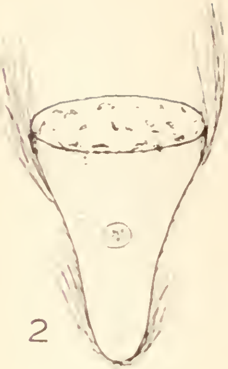
FIG. 6. A later stage in the closing of the cavity.

FIG. 7. The cavity has closed and the wood particles have been ingested.

FIG. 8. Shows the cytoplasm of the posterior end flowed backwards for a considerable distance.

FIG. 9. One *Trichonympha* caught in the temporary cytostome of another and struggling to free itself.





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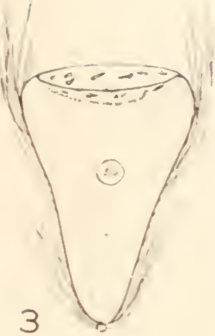

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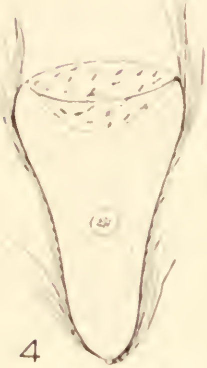

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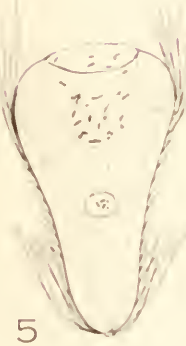

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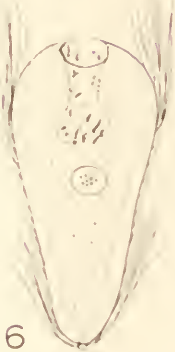

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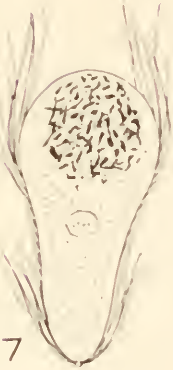

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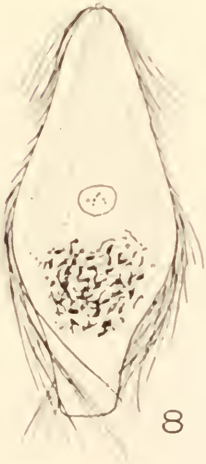

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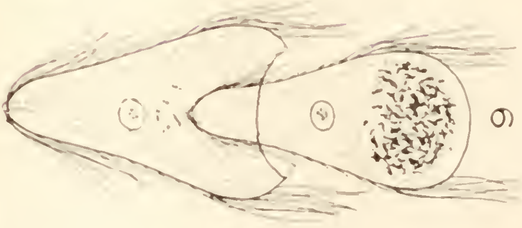

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L. R. CLEVELAND.

THE ABILITY OF TERMITES TO LIVE PERHAPS INDEFINITELY ON A DIET OF PURE CELLULOSE.

L. R. CLEVELAND.[1]

It seems quite certain from the work that has been done that the number of animals capable of using cellulose, along with other material, to any considerable extent is quite small indeed. This question, however, has been crucially investigated in few, if any, instances. For example, we are told that the goat can utilize a fairly high percentage of the cellulose content of its diet; the cow and the horse a smaller percentage; and man so small a quantity that it need not be considered. In most of the experiments which have been done on these animals, no distinction whatever was made between cellulose and hemicellulose, and the latter, which is perhaps more easily digested, was certainly present in a far greater quantity. In brief, we do not know whether cellulose can be used at all or not. The general belief is that it cannot, except perhaps in the case of some xylophagous insects. But the ability of these insects to utilize cellulose has been little investigated. Certainly, no animal—insect or what not—has been shown to be able to live on it. In the present paper the ability of an insect to maintain itself on a cellulose diet for twelve months and longer is demonstrated beyond question.

Most termites in nature feed solely on wood, which is always as much as 50 per cent. cellulose. This, then, coupled with the fact that they may be easily collected in great abundance and kept in the laboratory indefinitely, makes them ideal for the study of cellulose digestion.

Several thousand termites from two genera, *Termopsis* Heer and *Reticulitermes* Holmgren, were collected. *Termopsis* is the large Pacific Coast termite and is found in abundance in dead logs and stumps in California, Washington, Oregon, New Mexico and Arizona. *Reticulitermes* is a smaller form, with many species, some of which are present in almost any locality in the United States and many other countries. Material for this study was collected in Maryland. *Termopsis* belongs to the family Kalotermitidae and *Reticulitermes* to the family Rhinotermitidae. So, the two genera selected for experimentation are quite different in structure, habits, location, etc.

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Cellulose from two sources, cotton and wood, was used. That from cotton was the well-known Whatman filter paper No. 43, which is extracted in hydrochloric and hydrofluoric acids and in ether and contains .00006 per cent. ash. But cellulose prepared in this way is subjected to drastic treatment, which might render it more open to attack. In order to test this, fresh western yellow pine wood was made into sawdust, which was dried and ground so as to pass through a 50-mesh screen. This was extracted with alcohol and benzene and boiled under a reflux-condenser—Dore in a personal communication ('20) suggested the use and preparation of lignocellulose to me—for six hours, some in 5 per cent. HCl and some in 5 per cent. NaOH. In the material prepared in this manner, lignin and cellulose, with perhaps a trace of mannose, remain. But lignin has no food value, for when it alone is fed termites they do not live any longer than when they are starved. Hence we are dealing only with the cellulose content of this material, and results obtained by feeding it may be compared in every way with those obtained from feeding the cotton cellulose.

EXPERIMENTAL.

a. The Genus Termopsis.

The experiments were each carried out in two parts, which were duplicates except for food. In part one the food was lignocellulose and in part two it was cotton cellulose in the form of Whatman filter paper No. 43.

In each part of the first experiment, approximately 500 individuals, some soldiers but mostly nymphs, were placed in a large glass vessel which just fitted in a moist chamber. From time to time more food as needed was placed in the glass vessel and occasionally the fecal debris was removed. In the moist chamber around the outer edge of the glass vessel which contained the termites and their food, a small amount of water was added every ten days or so. In this way the food was constantly kept sufficiently moist to make it wholesome and yet not so moist that the termites would be injured by the growth of molds on their fecal pellets.

In each part of the second experiment, 10 nymphs were placed in each of ten large size sputum jars, which were kept in a moist chamber. Moisture and food were added as in the first experiment. This experiment differs from the first chiefly in the size of the vessel which contained the termites and in the number of termites which were placed in each vessel.

In each part of the third experiment, approximately 100 nymphs with food were placed in a liter Erlenmeyer flask. This flask was connected by a capillary tube to a similar flask which contained a little water. The air and water were changed every 60 or 70 days, but the flask which contained the termites was never opened, for sufficient food was added when the experiment was started to last to date—one year—and longer.

b. The Genus Reticulitermes.

This genus contains many individuals—half the colony at least—of the true worker caste, while *Termopsis* does not have any true workers. In each experiment, workers, soldiers, and nymphs were used.

Three experiments were carried out, each in two parts, using lignocellulose in part one and cotton cellulose in the form of Whatman filter paper No. 43 in part two, just as with *Termopsis*, except that in the third experiment four flasks, with approximately 500 termites in each, were used.

RESULTS.

So far as it is possible to determine, these termites on a pure cellulose diet have behaved in every way exactly as the controls, that have been given a wood diet and kept in the same manner. No difference whatever has been noticed between those that received a diet of cotton cellulose (Whatman filter paper No. 43) and those that received a diet of wood cellulose (lignocellulose). The drastic treatment to which the cotton cellulose is subjected during preparation seemingly, then, does not render it more open to attack. At any rate, the lignocellulose, subjected to much less drastic treatment, is just as open to attack.

Everything that has been observed in termites in nature during a year's time, has been observed in these cellulose-fed termites. Many of the nymphs have become sexually mature young adults and have laid a very large number of eggs, which have hatched normally. Larvæ from these eggs have grown even more rapidly than those from the eggs of some of the wood-fed controls. In all the experiments, the larvæ now present are greater both in number and in actual body weight than those individuals which were present in the beginning. In other words, the total weight of the colony is now more than twice what it was when the experiment was begun. This increase which has taken place on a cellulose diet is as great as that which has taken place on a wood diet. No deaths have occurred. Molting and the formation of winged adults have been observed in each experiment. Cellulose, then, in every noticeable way, has been as nutritious, so far (12 months), as the normal diet of wood.

Discussion.

If these insects can maintain themselves in a perfectly normal manner indefinitely on a cellulose diet, they must be able in some way to fix atmospheric nitrogen, which they use in manufacturing proteins; or else, contrary to the current opinion, they must be able to transform carbohydrates into proteins. These two possibilities are now being investigated from many angles.

It should be mentioned, for the benefit of those who have not seen the earlier papers of the writer ('23*a*, '23*b*, '24*a*, '25*a*, '25*b*) on the symbiosis between termites and their intestinal protozoa, that termites from which the protozoa have been removed by either of the three methods (incubation, starvation, oxygenation) lose the ability to live on cellulose or on wood. However, when the protozoa are restored, *i.e.*, when the defaunated termites are reinfected, the ability to live on cellulose or on wood is regained. The teeming menagerie of intestinal protozoa which the termites harbor, then, either digest the cellulose *in toto* or else play a very important part in its digestion.

ADDENDUM.

It is now eighteen months since these experiments were started. The termites appear perfectly normal in every way. Thousands of eggs have been laid during the past week, and several winged forms have been produced. In some cellulose-fed artificial colonies which contained ten adult individuals in the beginning more than two hundred half-grown individuals are now present. Thus, the weight of these colonies has increased more than forty times on a diet of cellulose.

Several attempts have been made to determine whether or not atmospheric nitrogen is being fixed. The respiratory quotient has been measured and, as would naturally be expected on a carbohydrate diet, has been found to be practically 1/1. When termites (*Termopsis*) are confined in air with barometric changes being noted and temperature being kept constant, a negative pressure is very soon developed. This indicates that nitrogen is being fixed, but analyses of air samples taken from tubes where the negative pressures have developed have shown very little, if any, change in the nitrogen percentage.

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BIOLOGICAL BULLETIN

THE FEEDING HABIT OF TERMITE CASTES AND ITS RELATION TO THEIR INTESTINAL FLAGELLATES.

L. R. CLEVELAND.[1]

More than 100 genera and approximately 1,200 species of termites are known; and each species is usually composed of five castes, with male and female individuals in each. Three of these castes, commonly referred to now as *first*, *second*, and *third forms*, are responsible for the reproduction of other individuals like themselves and for two other castes, *workers* and *soldiers*, which, although they possess reproductive organs, have given up the reproductive function (if they ever possessed it).

Nearly all the observations and experiments in the present paper have been carried out on one of the most common North American termites, *Reticulitermes flavipes* Kollar, whose castes (Thompson and Synder, '20) may be briefly described as follows:

(1) *First form*, which has three well-defined phases of development: (*a*) the nymphs (Figs. 1, 2), with long wing pads, creamy white body 1.3–1.4 mm. long, light brown eyes; (*b*) the winged adults, with long wings, dark brown body 6 mm. long, and black eyes; (*c*) the older males and females (Fig. 3), with enlarged abdomens and the scales of the shed wings, body 7–14 mm. long.

(2) *Second form*, which, like the first form, has three well-defined phases of development: (*a*) nymphs (Figs. 4, 5), with short wing pads and colorless body and eyes; (*b*) the young adults, with short scaly wing vestiges, straw-colored or grayish body 6–7 mm. long; (*c*) the older adults (Fig. 6), with wing vestiges, enlarged abdomen, body length 7–12 mm.

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(3) *Third form*, which also has three developmental phases: (*a*) the nymphs, wingless, with white head and body, and eyes that are invisible in the living or unstained specimen; (*b*) the young adults, no wing vestiges, head and body white, 7–9 mm. long.

(4) The *worker* (Fig. 7), wingless, with grayish abdomen, only two developmental phases, *i.e.*, nymphs and adults, salivary glands small and very little fatty tissue is present in the body, blind.

(5) The *soldier* (Figs. 8, 9), wingless, with elongated head covered with thick yellowish chitin, mandibles very large, long, dark brown, slender and curved, abdomen shorter than in other castes and more flattened, nymphs and adults only, no post adult growth as in reproductive castes.

Thompson ('17) showed that the newly hatched nymphs of *Reticulitermes flavipes*, 1.1 mm. long, although externally all alike, could be differentiated by their internal structures into two distinct types, namely, (*a*) reproductive nymphs, from which the three fertile adult castes develop, and (*b*) the worker-soldier nymphs, from which the sterile adult castes develop. By the time reproductive nymphs had attained a body length of 1.3–1.4 mm. they could be differentiated by their internal characters into nymphs of the first form and nymphs of the second form, which developed, finally, into the two respective reproductive adult castes. Soldier and worker nymphs could be differentiated internally by the time they had attained a body length of 3.75 mm. Nymphs of the third form could not be differentiated until a body length of 4 mm. was attained.

These five castes occur in most termites. Known exceptions are: the third form occurs in few, if any, species of the family Termitidae; a true worker caste is not present in the genera *Termopsis* Heer and *Neotermes* Holmgren, but a large-headed worker-like reproductive form is present; two genera, *Kalotermes* Hagen and *Cryptotermes* Banks, have no worker caste; the genus *Anoplotermes* F. Müller has no soldier caste; some species have as many as three types of soldiers, which, if counted as castes, make these species have seven castes, provided the five described above are all present.

Grassi ('93) calls the first forms "true" or "perfect" insects, or "royal forms." The second and third forms he calls "substitute" and "complemental" forms, which forms, he thinks, are always ready to take the place of the royal forms in case of need. This author believes the castes are a product of environment and special feeding; what environment and special feeding, he does not consider. Bugnion ('12, '13) and Imms ('19) believe the castes are a product of the germplasm. The field observations that have been made (Snyder, '15, '16) support this contention. So do the morphological studies of Thompson ('17). But we really *know* absolutely nothing about what produces the castes. The question is badly in need of study. Jucci ('20),[1] who recently announced the discovery of a particular diet which brings about caste production, has added nothing but confusion, subtlely clothed in high-sounding phraseology, to the origin of a most interesting phenomenon.

From the description that was given of the castes, it may be clearly seen that very great morphological differences exist within a termite species, *i.e.*, the castes are quite distinct. It also seems likely that physiologically the castes are equally distinct.

The writer ('23*b*, '24*a*, '25*b*) has definitely shown that the removal of the intestinal protozoa from at least two genera of termites (*Reticulitermes* and *Termopsis*) makes it impossible for them to live on their normal diet of wood. He ('23*a*) has also shown that if termites harbor protozoa, they must feed on wood or cellulose. In the present paper a study of the various castes has been made in order to determine whether or not what is true for termites in general is also true for each caste throughout its life-cycle. In addition to this, some data on the physiological differentiation of castes and the relation of the castes to each other have been obtained.

EXPERIMENTS AND OBSERVATIONS.

Forty to fifty colonies of *Reticulitermes flavipes* were collected and have been kept for the past three years in the laboratory where they have been carefully observed. More than 300 second and third form young adults and 150 soldiers have been isolated from these colonies and have been used in experiments. And several hundred have been kept for observations. Thousands of workers and first forms have figured in experiments and observations.

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[1] After this paper had gone to press a voluminous monograph by Jucci appeared, in which much attention is given to considerations of minor importance. A good beginning has been made, but the problem of the origin of termite castes has not been solved.

One experiment, which was later duplicated five times with the same result each time, was carried out as follows: Five individuals of each of the five castes, workers, soldiers, third forms, second forms, and first forms, were placed in each of five large size sputum jars with food. These jars were kept in a moist chamber, so that the moisture, as well as temperature and light, would be identical in all the experiments. Experience has also shown that this is the best way to keep a colony of termites normal in the laboratory. Another experiment, which was duplicated three times with the same result each time, was carried out in the same manner as the experiment just mentioned except that the termites were kept in screw-top jars which were not placed in a moist chamber. In both of these experiments the second forms, the third forms, and the soldiers were all dead, in every instance, within three to four weeks, while the first forms and workers were able to live indefinitely.

Many observations were made daily throughout the course of these experiments and no castes except the workers and first forms were ever seen to take food. At various intervals several individuals were killed and their intestinal contents were carefully examined microscopically, with the result that no wood particles were ever present except in the workers, first forms, and soldiers—just how the soldiers came to have wood particles in their intestines and yet were not able to live will be made clear later. It seems quite evident, indeed, then, that the young second and third form adults do not feed on wood; that they get their nourishment perhaps in the form of salivary secretions from the xylophagous members of the colony.

Why do some castes die when placed by themselves? Is it because they do not take food? To test this point further, twenty experiments were carried out, ten where one worker was placed in a vial with two second form individuals and ten where one worker was placed in a vial with two soldiers. Under this condition, the second forms and soldiers were able to live indefinitely. Thus one worker can at least support itself and two soldiers or two second forms. How much more a worker can do, was not determined.

Why do the second and third form young adults not take food when placed by themselves? A very large number of individuals of the second and third form castes were examined carefully for protozoa and in no instance were protozoa found after the final molt. In this connection it is interesting to note that every time any individual of any cast molts, its intestinal protozoa are lost, but in all molts, except the last one of the second and third forms, the protozoa are regained very quickly. Sometimes—though very rarely indeed—as the intestine slips off during the molting process, a portion of it is eaten before the protozoa die; but, as a rule, if an individual at the time of molting is placed to itself, it does not regain its intestinal protozoa and, as a consequence, dies within three weeks or thereabouts. But the first form regains its intestinal protozoa after the final molt. Why is it able to do this, while the second and third forms cannot? Obviously, one reason why the second and third forms cannot live by themselves after the final molt is because they have lost their protozoa. But this does not explain why they do not take food, for workers continue to feed after the protozoa have been removed from them experimentally.

Why and how are the protozoa lost? In an effort to throw light on this question, the second form was studied during the short period just before the final molt in which it can be distinguished externally from the first form. It was found that the protozoa gradually disappear during this period and that few, if any, are present at the time of the final molt. It must be noticed that there is also a dimunition in the protozoa in the first form during the period preceding the final molt, but not so great as in the second form, and the protozoa never disappear entirely. This progressive disappearance of the protozoa of the reproductive castes at this time is perhaps brought about by one or both of two things; namely, the salivary secretion which is taken during this period of rapid change and development destroys the protozoa and, as a consequence, wood-feeding must be given up; or, so much salivary secretion is taken that wood-feeding is thus made unnecessary and is, therefore, given up, in which case the protozoa die due to wood starvation, just as happens experimentally (Cleveland, '25*b*) when any normal wood-feeding termite containing a large number of protozoa is starved. In all three reproductive castes it is quite evident that much less wood is eaten at this time; but in the first form the wood diet is not entirely given up; it may be curbed greatly, though never supplanted, by the salivary diet. It is interesting in this connection to note that the first forms eat much more wood shortly after the final molt and, because of this, the protozoa increase rapidly in number. They certainly receive no salivary secretion from workers for sometime if they leave (swarm) the parent colony to start a new colony, and nearly every one, if not every one, leaves or is killed. Their only food is wood until they rear workers to furnish them salivary secretions again, and when this is done, they again progressively cease to eat wood, finally giving up the habit entirely, at which time they lose all their protozoa and become dependent on the xylophagous members of the colony for the rest of their lives. It is interesting here to note that instinctively this dependence, which is perhaps inevitable, is well taken care of or looked forward to, because mostly workers are reared in the first brood of such a reproductive pair. But would they become dependent if not allowed to rear workers, that is if the larvæ were killed or taken from them?

We have already said that one reason why the second and third form young adults die when placed by themselves is because they lose their protozoa, and the protozoa are lost because these forms do not feed on wood, the second and perhaps more fundamental reason why death results when such individuals are isolated. But why do they not eat wood? According to Thompson and Snyder ('20) the jaw muscles and many others, particularly those in the head, of the first form degenerate during the post-adult stage. "This degeneration of the jaw muscles," they state, "is due to the fact that the reproductive forms are now fed by the workers on partly digested food and no longer masticate wood as they were compelled to do before the first broods of workers were raised." These authors also observed that the jaw muscles in the second and third forms in the post-adult stage had degenerated, though they did not state when the degeneration occurred. If, in the first forms, it occurs, as they state, when workers supply them with "partly digested food," thus making the eating of wood unnecessary, may we not reasonably assume that in the second and third forms it also occurs when partly digested food is supplied them by workers and wood-feeding is permanently given up, that is at the time of the final molt or thereabouts, considerably earlier than in the first forms. If this is true, we know why the second and third form young adults when experimently placed by themselves do not eat wood and die; their jaw muscles have degenerated, thus making it impossible for them to eat wood, and they can only survive when fed by workers.

But what causes the jaw muscles to degenerate? Thomson and Snyder ('20) think it is due to disuse, brought about when the salivary diet takes the place of the wood diet. If this is true, then, the second and third forms must get more salivary secretion or partly digested food than the first form, since they lose the ability to eat wood and, of course, their protozoa that digest the wood for them, much earlier in life, at least two years earlier. If it is true that salivary secretion brings about a degeneration of the jaw muscles, why are the reproductive forms fed a salivary diet? Certainly not to make the jaw muscles degenerate, for this is surely only a consequence of some deeper, underlying reason for feeding a salivary diet to the reproductive forms. In other words, if the jaw muscles do not degenerate except through disuse, the salivary secretion is perhaps a necessity and may play a vital part either in accelerating or changing the course of development. On the other hand, if the jaw muscles degenerate in the absence of a salivary diet, that is not because of disuse, it may be that the function of such a diet is simply to take the place of a wood diet which becomes impossible. If this is true, the question, why do the jaw muscles degenerate, is perhaps as vital as caste production itself, which, if a result of food, would perhaps be stopped, or at least held in abeyance to some extent, if individuals were isolated very early. A few attempts have been made to get second forms early in the gradual decline of wood-feeding, which occurs simultaneously with a progressive dimunition in the number of protozoa. It was found that such second form individuals when isolated early can live by themselves longer than if allowed to remain with workers for a while and isolated at a later stage of development, thus showing that at the time of the earliest isolation they had already begun to gradually lose the ability to maintain themselves and that they lose it more quickly when they remain with workers who fed them. Thus the decline in wood-feeding occurs anyway regardless of the time of the isolation, although more slowly seemingly when workers are not present. However, this experiment does not mean much, for long before the external differentiation has occurred which makes the distinction between the second and first form possible, several quite noticeably distinct internal microscopic differentiations had already occurred, thus showing that the differentiation cause had its origin much earlier and had been operating for sometime before the attempt to arrest it was made. Perhaps the workers could distinguish them and had been feeding them. To settle the question in this way, one should begin the isolation at an earlier stage. But these microscopic differences just referred to are only distinguishable in *Reticulitermes* after fixation and staining. Possibly in other genera the task will be less difficult, and we may be able to determine definitely what effect, if any, a salivary diet has on caste production, whether the decline in wood-feeding is caused by the salivary diet or whether the salivary diet has to be substituted for the wood diet after the jaw muscles have degenerated.

Since the second and third form young adults cannot live by themselves, it, of course, follows that they cannot start new colonies in the absence of workers. Incidently, the fact that they do not harbor protozoa shows beyond question, regardless of the fact that they cannot eat wood, that they never start new colonies in the absence of workers. This would be true just the same even if the protozoa were not absolutely necessary to their existence, because if new colonies were started by second and third forms, these colonies would not have protozoa in any of their castes. No such colonies have ever been found; consequently workers must be present when these forms head a colony.

It should be mentioned here that in the genus *Termopsis*, which has no true worker caste, it appears from the observations that have been made that the ability to eat wood is not lost in the second and third forms. But the observations on this genus are really too few to warrant a conclusion further than that these forms certainly do not lose their protozoa and the ability to eat wood as early as they do in *Reticulitermes*, which has workers. If the degeneration of the jaw muscles occurs in the reproductive forms when and because workers supply them with a salivary or partly digested diet, we should not expect to find degenerate jaw muscles and enlarged dependent reproductive forms in those genera where workers are not present, unless, of course, the young nymphs play the same rôle that workers do in other genera.

We will now return to the soldier caste and take up the question, why are adult soldiers unable to live by themselves? Simply because their very large and heavily chitinized mandibles (Fig. 9) will not permit them to eat wood. They, too, like the second and third forms, lose the ability to eat wood but from a growth process rather than one of degeneration. Before their mandibles grew so large, they could eat wood and could live by themselves. Could these mandibles be altered by a change in diet? Did a diet produce them? We cannot answer either question.

Soldiers can digest wood, for they have protozoa in their intestines to do it for them, but are unable to eat it. They lose the ability to eat wood without losing their protozoa, for they get protozoa, just as all other castes do, very early in life from the ani of the xylophagous members of the colony. As we have said, owing to their very enormous and highly specialized mandibles (Fig. 9), they cannot chew wood, but they manage to ingest proctodael wood particles—partially digested perhaps—which have passed through the alimentary canal of those members in the colony capable of chewing wood. Their intestines are considerably smaller and they harbor a smaller quantity of protozoa than workers of the same size. Soldiers, then, are not as dependent, in one sense, on workers or permanent wood-chewing members of the colony as the second and third forms are, for they do not require totally predigested food, such as the salivary secretions upon which the second and third forms probably feed entirely after the final molt.

SUMMARY.

All results were obtained from laboratory colonies which have been carefully studied during the past three years. Many of these results have been verified by field observations.

At every stage in the life-cycle of any caste where wood is eaten, protozoa are present. When wood is not eaten or obtained in some way, protozoa are never present.

Second and third form young adults have lost the ability to eat wood. The protozoa in these castes disappear concomitantly with the loss of the ability of their host to feed on wood, and by the time the wood-eating ability is lost, they have all disappeared. This occurs about the time of the final molt and is perhaps brought about by the feeding of salivary secretions which take the place of the wood diet. In all castes, the protozoa are lost during molting, but they are soon regained, except in the final molt of the second and third forms, in which forms they are never regained because, owing perhaps to the degeneration of their jaw muscles, these forms have lost the ability to eat wood. What causes the jaw muscles to degenerate is not definitely known. It may be inherent in these castes, as much a part of them as anything else. If it is, then the salivary-feeding is hereby made necessary and takes the place of the wood diet when the jaw muscles degenerate. But a more plausible possibility is that these forms are fed so much salivary secretion that they cease to feed on wood and because of this their jaw muscles degenerate through disuse, and thus the ability to feed on wood is lost forever.

The first form and the worker always eat wood, except in the post-adult stage of the life-cycle of the first form, where it, too, after having attained an old age loses the ability to eat wood and becomes dependent on the workers and young undifferentiated nymphs (when present) which it has reared. It is noteworthy that mostly workers are raised in the first brood.

Adult soldiers, owing to their large mandibles, cannot eat wood (cannot chew it), though they obtain it, together with protozoa from the ani of the xylophagous members of the colony. Soldiers, like workers, harbor protozoa throughout their life-cycle. Young soldiers (soldier nymphs), before they obtain the large mandibles, can chew wood for themselves. So can the second and third forms, during early life.

A caste which cannot eat wood, or, thinking in terms of the protozoa, a caste which does not harbor protozoa, cannot live by itself. Such individuals are dependent on the wood-eating members of the colony for support; consequently adults of the second form, third form, and soldier castes must be supported by other members of the colony. But the soldiers, in one sense, are not as difficult to support as the second and third forms, since they can digest for themselves the partially digested woody material which has passed through the alimentary canal of the xylophagous members of the colony before they receive it; while the second and third forms, since they feed exclusively on the salivary secretions, must subsist entirely on predigested food.

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EXPLANATION OF PLATE I.

FIGS. 1–9. The Castes of *Reticulitermes flavipes.*

FIG. 1. Nymph of first form, with long wing pads, creamy white body from 1.3–1.4 mm. long, light brown eyes.

FIG. 2. Side view of Fig. 1.

FIG. 3. Post adult first form queen, with enlarged abdomen, the scales of the shed wings, body length varies from 7–14 mm. This figure × 5.

FIG. 4. Nymph of second form, with short wing pads which never develop into wings, colorless body and eyes.

FIG. 5. Side view of Fig. 4.

FIG. 6. Post adult of second form queen, with wing vestiges, enlarged abdomen, body length varies from 7–12 mm. This figure × 5.5.

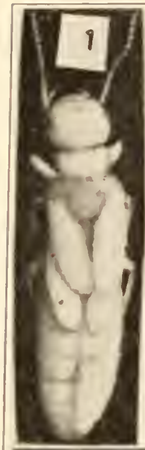
Note: The third form caste which for lack of space is not shown, has no wing vestiges, eyes cannot be seen except in stained material, is smaller having a body in the post adult stage of 7–9 mm.

FIG. 7. A group of workers enlarged three times.

FIG. 8. A group of soldiers enlarged three times.

FIG. 9. Mandibles of adult soldier which make the eating of wood impossible. × 16.

All figures (photographs) after Snyder.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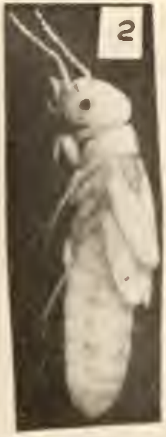
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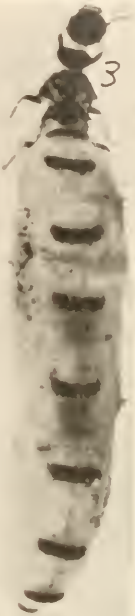
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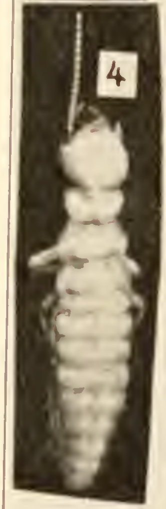
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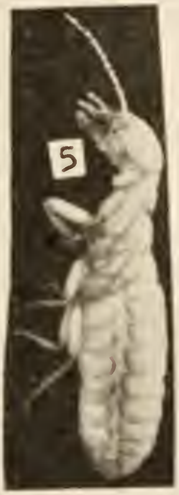
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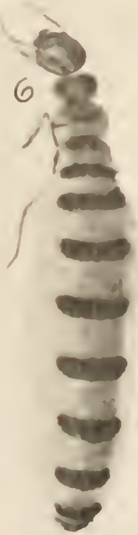
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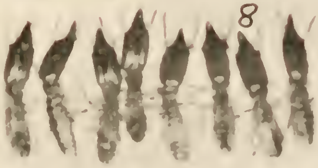
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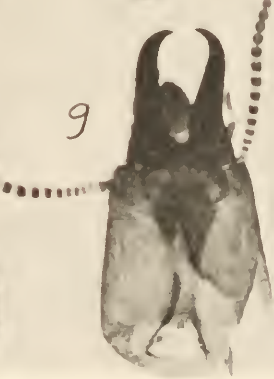
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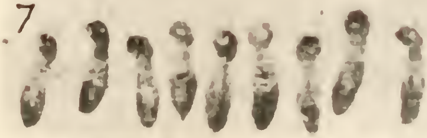
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L. R. CLEVELAND.

THE EFFECTS OF OXYGENATION AND STARVATION ON THE SYMBIOSIS BETWEEN THE TERMITE, *TERMOPSIS*, AND ITS INTESTINAL FLAGELLATES.

L. R. CLEVELAND.[1]

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INTRODUCTION.

In a previous paper ('24*a*) the writer reached the conclusion that xylophagous and protozoa-harboring termites are not able to live on their normal diet of wood after their intestinal protozoa are removed from them by incubation for 24 hours at 36° C. They die within three to four weeks if given a wood diet. When the protozoa are replaced, the termites concomitantly regain their ability to live indefinitely on a diet of wood or cellulose. Thus, the incubation, which removed the protozoa, did not kill the termites *per se*. Also, when termites from which the protozoa had been removed by incubation, were given a diet of fungus-digested cellulose, they were able to live indefinitely. Therefore, the ability to make use of cellulose (to maintain itself indefinitely on a wood diet an animal must be able to digest cellulose) seems to reside in the protozoa of the termites rather than in the termites themselves.

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In the present paper the protozoa have been removed from the large Pacific Coast termite, *Termopsis*, by two other methods, namely, starvation, and oxygenation, with the same results in each case as were obtained by incubation; the termites were not injured although the ability to live on wood disappeared simultaneously with the loss of the protozoa.

In addition to this, the relation of each species of protozoa to its host and to its neighbors or fellow protozoa, has been worked out by employing the various protozoal-removing methods separately and by combining them. Differential defaunation or the removal of some species of protozoa without affecting the others was thus attained.

Termopsis AND ITS PROTOZOA.

Three species of *Termopsis* are known. Two of them, *T. angusticollis* Hagen and *T. nevadensis* Hagen, can be distinguished only by an examination of their winged adults. In many of the colonies studied, no winged adults have appeared; consequently it has been impossible to determine whether all the experiments were carried out on one or both of these species. The winged adults which appeared in two colonies were *T. nevadensis*. Both species occur in the same localities in Oregon, California and elsewhere on the Pacific Coast and probably harbor an identical protozoan fauna.[1] But for these reasons, it seems most feasible to avoid specific determinations in this paper.[1]

The protozoa of *Termopsis* were first described by Kofoid and Swezy ('19) as follows: "In *Termopsis angusticollis* four different species of protozoans are invariably present." These they described as *Trichonympha campanula* (Fig. 1), *Leidyopsis sphaerica* (Fig. 2), *Trichomitus termitidis* (Fig. 3), and *Streblomastix strix* (Fig. 4). They state that "in addition to these there are usually present minute forms of two, sometimes three species of flagellates," which these authors did not study. These same small forms have been observed by the writer, but they are very small indeed, and few in number, and have been considered of no moment in the experiments that have been carried out.

[1] Later observations show that both species were used and that they harbor the same protozoa.

With two[2] exceptions (*Leidyopsis* and *Trichomitus*), no special effort has been made to verify the morphological studies of Kofoid and Swezy. However, as will be seen later, the writer has had occasion to observe many of these protozoa in almost pure cultures, which simplifies the matter of morphological details considerably, for unless one has really seen this overwhelming mass of squirming, wriggling, undulating protozoa which greet the eye when a termite's intestinal content is viewed under the microscope, one cannot form any conception whatever of the immense difficulty envolved in attempting to study in detail any of them except the very large and dominant genus *Trichonympha.* Of course, if one has a suitable medium in which to dilute the intestinal contents, these difficulties of observation are obviated greatly, but Kofoid and Swezy state that they did not find such a medium; hence it was difficult for them to make out the finer structures of the two smaller forms, *Trichomitus* (?) and *Streblomastix.*[2]

Leidyopsis, when millions of individuals are viewed in a suitable medium and unobscured by other protozoa, is not nearly so rounded as Kofoid and Swezy have figures from stained specimens (Fig. 2). This rounding up which they show is an abnormality of fixation. In living material it occurs also unless the observation is made in a suitable medium (Cleveland, '25*d*).

In the *Termopsis* material—most of which came from Oregon, though three colonies were obtained from California—which the writer has studied there is certainly a species of *Trichomonas* present; whether or not *Trichomitus* is also present it seems almost impossible to say, though all evidence indicates that it is not. For instance, in those hosts which were experimentally freed of *Trichonympha* and *Leidyopsis*, thus affording a wonderful opportunity to study *Trichomonas* (and *Trichomitus* too if present), an opportunity which Kofoid and Swezy did not have, an axostyle (the distinction between *Trichomonas* and *Trichomitus*, a genus which Swezy founded in 1915, lies chiefly if not entirely in the presence of an axostyle in *Trichomonas* and the absence of it in *Trichomitus*) can be seen instantly in 50 per cent. of the individuals exclusive of *Streblomastix;* with more study the number of individuals in which an axostyle is visible increases to 70–80 per cent.; with still more study after fixation and staining the percentage of individuals in which axostyles may be seen increases to 85–90 per cent., though never reaching 100 100 per cent. In other words, the number of individuals which at first glance would be diagnosed as *Trichomitus*, diminishes concomitantly with scrutiny of observation. Professor Kofoid when notified recently by letter of this finding and of the possibility that the species of *Termopsis* in California (although the protozoa in those colonies I examined were the same as in the Oregon *Termopsis*) might harbor *Trichomitus* while in Oregon they harbor only *Trichomonas*, wrote: "We find both *Trichomonas* and *Trichomitus* in the same hosts in California. We have regarded this *Trichomonas* as possibly the one described by Dogiel although we have not attempted to work it up." Professor Kofoid also kindly sent me slides which show an abundance of *Trichomonas*. However, in the papers of Kofoid and Swezy ('19), as noted by the quotations already given, no mention of the presence of *Trichomonas* appeared. This organism in *Termopsis* does not seem to me to be the same one which Dogiel [1] described as *Tetratrichomonas macrostoma* from *Rhinotermes* sp. of Uganda. The two hosts are widely separated and belong to two families; *Rhinotermes* Hagen belongs to the Rhinotermitidæ, and *Termopsis* Heer to the Kalotermitidæ. The species of *Trichomonas* in *Termopsis* has four anterior flagella (elsewhere the statement was made by the writer ('24*a*) that it had three anterior flagella, but more careful examination shows clearly that some individuals have four anterior flagella, which is perhaps the normal number), axostyle and undulating membrane, which place it definitely in the genus *Trichomonas*. I, therefore, describe it (Figs. 5, 6) as *Trichomonas termopsidis* sp. nov., found certainly in *Termopsis nevadensis* Hagen and probably in *T. angusticollis* Hagen.

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[2] *Streblomastix* has only four flagella. See addenda.

This is not the time nor the place for a discussion of the genera *Trichomitus* and *Trichomonas*. If *Trichomitus* is present —though I am inclined somewhat to doubt it—it has behaved the same way as *Trichomonas* in the starvation and oxygenation experiments. But, since it is not certain that *Trichomitus* is present or even exists, no mention of this genus will be made in these experiments.

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[1] Dogiel, V.; *Jour. ruse de Zoöl.* (Petrograd), 1.

EXPERIMENTS AND OBSERVATIONS.

a. Starvation.

Ten experiments, employing altogether approximately five hundred termites, were carried out as follows: The termites were removed from wood and carefully freed of all wood particles which they attempted to cling to. Then they were placed in large Petri dishes which were kept in moist chambers. In this way the amount of moisture which previous work had shown most desirable was constantly supplied. All the way from one to fifty individuals were placed in a Petri dish during starvation, with the same results in all instances.

When starved in this manner, *Termopsis* begins to lose its large, dominant and principal wood-ingesting protozoön, *Trichonympha* (Fig. 1), by the end of the third day, and by the end of the fourth day perhaps half the individuals of this genus originally present are dead, although few, if any, of the other genera of protozoa have died. By the end of the fifth day, some termites have lost all their *Trichonympha*, while others still retain a few slowly moving, apparently weak individuals. By the end of the sixth day, no *Trichonympha* can be found in any termites, and perhaps half the individuals of the next largest protozoan, *Leidyopsis* (Fig. 2), have died. And in a few termites perhaps all or nearly all of *Leidyopsis* may be dead, but this is exceptional. Also, by this time a few individuals of the next smallest genus, *Trichomonas* (Figs. 5, 6), may have died, but not many. If starvation is continued through the seventh day, some termites completely lose their infection of *Leidyopsis*, while others harbor a few individuals until near the end of the eighth day. After eight days of starvation, then, the two large protozoa, *Trichonympha* and *Leidyopsis*, have all disappeared entirely. If the starvation is continued, *Trichomonas* now begins to die rapidly and by the end of the tenth day nearly all the individuals of this genus are dead, though perhaps one to two per cent. of the total number originally present live sometimes four or five days longer. *Trichomonas* struggles along and dies more slowly than *Trichonympha* and *Leidyopsis*. All of these protozoa feed on the wood particles which their host has eaten, but *Streblo-*

mastix (Fig. 4) does not. It may be dependent on the other protozoa or on the termites for its nourishment. It is difficult to say just when *Streblomastix* begins to die since this genus is much smaller than either of the other three and in the normal or not-starved termite is greatly obscured by the countless thousands of larger individuals, thus making it difficult to determine accurately the normal number present; but the number has perhaps diminished some by the end of the tenth day and by the fifteenth day a great many have died, though not all; some of them, in fact, live almost as long as the termites—three to four weeks.

During starvation most termites are active and appear normal for about fifteen days. However, as soon as many of the protozoa have died, after six days, say, it is not necessary to make a microscopical examination of the intestinal contents to determine what has happened, for there is now much more fluid than formerly present and it looks very muddy—the difference is quite characteristic and cannot be mistaken.

Why do termites, when not given food (wood), lose their protozoa? Do the protozoa die of actual starvation, and most of them much more quickly than their host? Three experiments were carried out which perhaps throw some light on this question. When termites are fed cellulose instead of wood for several months before being starved, and then are cellulose-starved, they lose their protozoa more slowly. For instance, it takes them at least one to two days longer to lose *Trichonympha*. The writer ('25*c*) has shown elsewhere that this termite (*Termopsis*) can live for more than a year and perhaps indefinitely in a perfectly normal manner on a diet of pure cellulose and may it not be true that when it is fed nothing but cellulose (wood is about 50 per cent.) for some time before being starved that it really has more food in its intestine for the protozoa when starvation is begun and for this reason the protozoa are able to live longer? If so, this indicates that when *Termopsis* is wood-starved that its intestinal protozoa, particularly *Trichonympha*, *Leidyopsis*, and *Trichomonas*, die of actual starvation long before their host. We should expect the protozoa to die first since they digest the wood for themselves and their host. And when they die, they perhaps give themselves as food to their host, which is thus enabled to live considerably longer than its protozoa. In nature, termite protozoa may aid their host by giving themselves as food. It is not known how long the life-cycle of these protozoa is, but, if it is not longer than that of the parasitic protozoa that have been cultivated in artificial media from a single individual, countless millions of them must die daily in a single termite.

b. Oxygenation.

It has been fairly common knowledge for some time that oxygen in rather excessive or abnormal amounts is toxic for many, if not all, forms of animal life. Realizing there must be very little oxygen in the environment of intestinal protozoa, I concluded that they might for this reason be more sensitive to it than their host in an atmosphere of approximately 20 per cent. oxygen. Accordingly, it was decided to determine whether oxygen was more toxic for intestinal parasites than for their host. Obviously, for many reasons, termites are far superior to any other insect and perhaps any other animal for such a study. Workers, soldiers, and nymphs of the reproductive castes, can be counted on to have an infection of 100 per cent., approximately the same in all individuals of the same size and age. And there are millions of very large, active and highly specialized flagellates in a single insect. In fact, nearly half the body weight of the insect is made up of these protozoa. Where, then, could a better opportunity be found to study the effect of oxygen on intestinal flagellates? The termites are easily kept in the laboratory and will live almost indefinitely in tightly corked vials and flasks, for they can stand a very high percentage of CO_2.

These experiments were begun with a different end in view from the use that is made of them in this paper, for it was not thought that all the intestinal protozoa could be removed without injuring the termites too, and still less was the possibility contemplated that oxygen might entirely remove some genera of these flagellates before killing others. The other subject, that of the toxicity of oxygen proper, is being studied now and will be taken up in detail in a later paper.

It was found that if termites (*Termopsis*) were placed in fairly pure oxygen at one atmosphere pressure that all protozoa belonging to the genus *Trichomonas* (Figs. 5, 6) were killed within 24 hours and had disappeared from the termites' intestines, while *Trichonympha* and *Leidyopsis*, the two largest genera, and *Streblomastix*, the smallest genus, remained practically unaffected. Of course a few others were probably killed, but not many. The termites were not affected in the least by the oxygen. However, we should not expect them to be for many animals can live in an oxygen atmosphere at this pressure for a much longer period. The surprising thing is that the oxygen kills the protozoa so quickly and removes one genus completely long before the others.

When the termites were confined to the oxygen atmosphere for more than 24 hours, *Trichonympha*, *Leidyopsis* and *Streblomastix* began to die, though not all individuals of these genera were dead until about 72 hours. Sometimes they were dead a little earlier than this and sometimes a few hours later, the variation probably depending on the percentage of oxygen in the particular flask or vial containing them. The oxygen which was used was obtained by heating C. P. $K_2Mn_2O_8$ and was washed in NaOH before being run into the flasks and vials where it displaced most of the air. No effort was made to determine the percentage of oxygen in such an atmosphere, which must have varied considerably at times. Known percentages of oxygen at one and at more than one atmosphere pressure are now being used in the work on toxicity of oxygen for intestinal protozoa. The significant fact is that in all of more than 75 experiments which were carried out the protozoa were all removed in approximately 72 hours. In these 75 experiments, more than 1200 termites were used and none of them, in so far as could be determined by careful observation, ever suffered any ill effects from the oxygen. They easily live eight to ten days in an oxygen atmosphere which kills their intestinal protozoa in three days. No effort was made to determine just how long they would live, for after their intestinal protozoa have been taken from them, they cannot live more than three to four weeks in air.

c. Oxygenation and Starvation.

It was noticed in the starvation experiments that *Trichonympha* disappeared entirely after the termites were starved six days and that *Leidyopsis* had disappeared entirely by the end of the eighth day of starvation. In the oxygenation experiments, *Trichomonas* disappeared entirely within 24 hours. It was obvious, then, that a combination of these two methods for removing the protozoa would yield interesting results. Accordingly, ten experiments, using approximately 400 termites, were carried out as follows: The termites were oxygenated, as in previous experiments, for 24 hours. This removed all protozoa belonging to the genus *Trichomonas*. About 100 of these termites were starved for six days, and 100 for eight days. In this manner, the first 100, or those individuals which were starved six days, were freed of *Trichonympha*, and the second 100, or those individuals which were starved eight days, were freed of *Trichonympha* and *Leidyopsis*. Thus, in those individuals that were starved eight days, *Streblomastix* only remained, while in those individuals that were starved six days, *Leidyopsis* and *Streblomastix* remained.

d. Wood-feeding after Intervals of Starvation and Oxygenation.

By starvation and by oxygenation and by a combination of starvation and oxygenation we have seen how it is possible to shift the protozoa about almost any way we wish. For instance, we can take out *Trichonympha* by starving six days and leave *Leidyopsis*, *Trichomonas* and *Streblomastix* uninjured; by starving eight days we can remove *Trichonympha* and *Leidyopsis*, leaving *Trichomonas* and *Streblomastix*, and then by oxygenating 24 hours we can remove *Trichomonas*, leaving only *Streblomastix*; or we may oxygenate first and remove *Trichomonas* which will leave *Trichonympha*, *Leidyopsis* and *Streblomastix*, and then if we starve these individuals for six days we have *Leidyopsis* and *Streblomastix* remaining; and by oxygenating for 72 hours all protozoa are removed. By this crisscross procedure we may obtain termites with five[1] protozoal combinations and protozoa-less termites as follows: (1) *Leidyopsis*, *Trichomonas*, *Streblomastix;* (2) *Trichomonas*. *Streblomastix;* (3) *Streblomastix;* (4) *Trichonympha*, *Leidyopsis*, *Streblomastix;* (5) *Leidyopsis*, *Streblomastix;* and (6) no protozoa. Thus a wonderful opportunity is afforded for studying the relation of each of the four genera of protozoa to its host and to its neighbors or fellow protozoa, for none of these six groups of termites was injured in the least by the methods employed in removing the protozoa. Each group would feed on wood just as it did before the protozoal alterations were made.

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[1] Two more combinations have been obtained recently. See addenda for a tabulation of all combinations.

Each of these six groups was now fed wood and kept to itself in the same environment of temperature, moisture, and light. Each group contained about fifty individuals. Controls, or termites that had not been treated in any way, were also kept with these five groups. The results of feeding wood to each group may be briefly stated as follows:

(1) *Termites with Leidyopsis, Trichomonas and Streblomastix.*—In normal termites in nature, *Trichonympha* for some reason is perhaps 1000 times as numerous as *Leidyopsis*, and this ratio is fairly constant, although we do not know what makes it so. *Trichonympha* is the dominant genus in size at least and probably in number too—*Trichomonas* and *Streblomastix* may sometimes be as numerous as *Trichonympha*, but they are much smaller. A most interesting thing happens to *Leidyopsis* when its dominant neighbor, *Trichonympha*, is killed; it multiplies rapidly, soon increases very greatly, indeed, in number, and in 20–30 days has filled up the space made vacant in the termite's intestine by the removal of *Trichonympha*. This condition remains permanently and the group of termites is able to live indefinitely on a wood diet. *Leidyopsis*, then, is not only able to take the place of the dominant *Trichonympha* in number but can also take its place as the chief symbiont. As we shall see when we come to study the group of termites with *Trichomonas* and *Streblomastix*, or the group with *Streblomastix*, *Trichonympha*, in nature, is by far the most important symbiont. We know this, even though we were not able to get a pure culture of this genus, because *Leidyopsis* is present in too small a number to be of much importance and *Trichomonas*, as group (3) shows, is not of very great moment as a symbiont. So, in nature, *Trichonympha* is of most value to termites, because for some reason it is dominant over *Leidyopsis*; although, under experimental conditions, *Leidyopsis* can become of as great value to its host as *Trichonympha* is in nature.

(2) *Termites with Trichomonas and Streblomastix.*—When

Leidyopsis, although it is usually never present in nature in nearly such large numbers as *Trichomonas* and *Streblomastix*, is removed the same thing happens to *Trichomonas* that happens to *Leidyopsis* when *Trichonympha* is removed, namely, *Trichomonas* multiplies rapidly and increases very greatly in number for about 30 days, but it never completely fills the intestine of its host. These termites are active for approximately 60–70 days on the average, but after this period many of them die, although some of them live considerably longer and a very small percentage may be able to live indefinitely. However, under the present conditions, we may conclude that the symbiosis between *Termopsis* and its intestinal protozoa is very greatly damaged by the removal of *Trichonympha* and *Leidyopsis*. *Trichomonas*, under these experimental conditions, is undoubtedly of some value to its host, though certainly not as much as either *Trichonympha* or *Leidyopsis*. It is able to keep its host alive for at least 40–50 days, for when it is removed, as we shall see in group (3), the termites die 40–50 days earlier. It is possible that *Trichomonas* in this case has to support *Streblomastix*, as well as its host, for *Streblomastix* cannot live alone as group (3) shows. *Streblomastix* now, as was not the case when only *Trichonympha* had been removed, increases in numbers considerably, and, if a method for removing it without removing *Trichomonas* at the same time were available, *Trichomonas* might then be able to keep practically all of its hosts alive indefinitely. This, however, being a possibility which has not been tested, more work must be done before we can speak with exactness upon the precise ability of *Trichomonas* to keep its host alive.

(3) *Termites with Streblomastix.*—*Streblomastix* does not increase in number; on the contrary, it gradually diminishes, and its host dies within three to four weeks, just as when all protozoa are removed. We conclude, then, that *Streblomastix* is not a symbiont, for it does not seem to be of any value to its host. Incidently, this protozoön, unlike the other three genera, does not ingest wood particles from the intestine of its host, which also suggests that it plays no part in digesting food for its host. *Streblomastix* may depend on the other protozoa for its support.

(4) *Termites with Trichonympha, Leidyopsis, and Streblomastix.*—These termites are able to live indefinitely and it is

not possible to note any change in their protozoa. Perhaps *Trichonympha* takes the place of *Trichomonas*, but if this occurs, it cannot be detected because there are so many *Trichonympha* anyway. The loss of *Trichomonas* is of no consequence.

(5) *Termites with Leidyopsis and Streblomastix.*—*Leidyopsis* multiplies in this group as in group (1), and perhaps takes the place of *Trichomonas* as it does of *Trichonympha*. These termites, as those in group (1), are able to live indefinitely. Since *Streblomastix*, as group (3) shows, is of no value to its host, we may conclude that *Leidyopsis* alone, without the modicum of assistance from *Trichomonas* such as it received in group (1), is able to keep it shost alive indefinitely.

(6) *Termites with No Protozoa.*—These termites eat wood just as those in the other five groups, but are not able to live longer than three to four weeks. The inability to maintain themselves on their normal diet of wood is caused by the removal of the intestinal flagellates, particularly *Trichonympha* and *Leidyopsis*, from them.

Discussion.

Intestinal protozoa must live in an environment with a smaller percentage of oxygen than their hosts, and should, therefore, experience the greater difficulty when the oxygen environment of the host is raised from 20 to 100 per cent., provided, of course, the oxygen percentage of the parasite's environment does not increase correspondingly with that of its host. For instance, if there is normally, say, 1 per cent. of oxygen in the parasite's environment and 20 per cent. in that of the host, when the host is placed in an atmosphere of 100 per cent., the percentage of the host's environment is thus increased only five times, while that of the parasite is increased eighty times. On the other hand, if the oxygen percentage of the parasite's environment increases correspondingly to that of its host, or, as in this case, five times, then the parasite would be in 5 per cent. oxygen when its host was in 100. When termites are placed in 100 per cent. oxygen, the oxygen percentage of their parasite's environment may be increased much more than their own, and the parasites are killed, just as any animal would be with so great an oxygen increase. If this is true, the parasites can undergo as great change in oxygen as their host, and oxygen is really not any more toxic for them than for their host. They die, then, while their host is uninjured, because the oxygen percentage of their environment increases many times more than that of their host.

In larger animals with a different system of respiration, the oxygen percentage of host and parasite may increase correspondingly, in which case it would be impossible to kill the parasites of, say, a vertebrate by confining it in an oxygen atmosphere without at the same time killing the vertebrate, unless, of course, oxygen is actually more toxic for the parasites. At any rate, other parasites may be killed by the use of oxygen if we can develop a method of getting it to them.

When termites are starved, their largest protozoa die first, but when they are oxygenated, their next to the largest one dies first. What is the cause of this? The larger and more active ones may require more food than the smaller ones, and for this reason starve more quickly, if starvation is the actual cause of death. Or it may be that the smaller ones are partly nourished by their host or by their larger protozoan neighbors. In the case of oxygenation, the smaller ones may die first because of the higher ratio of surface-volume exposure which they have.

One interesting problem which this study brings out is, what maintains the fairly definite ratio between the four genera of protozoa? The host may produce a reaction product for each genus which inhibits its multiplication beyond a certain point. However, this does not seem very likely, for when *Trichonympha* is removed, *Leidyopsis* takes its place, and when *Leidyopsis* is removed, *Trichomonas* partly takes its place. The protozoa may inhibit the reproduction of each other beyond a certain point. And another possibility is the question of struggle for food which must go on where such a large number of protozoa are present.

Summary and Conclusions.

The termite which was used in these experiments belongs to the genus *Termopsis*. Very probably two species, *T. nevadensis* Hagen and *T. angusticollis* Hagen, have been used. These two species are so nearly alike that they can be distinguished at present only by a study of their winged adults, which were not present in most of the material used. The two species probably harbor an identical protozoan fauna.

Four genera of protozoa are invariably present in these termites. These in order of size are: *Trichonympha* (Fig. 1), *Leidyopsis* (Fig. 2), *Trichomonas* (Figs. 5, 6), and *Streblomastix* (Fig. 4). Kofoid and Swezy ('19) claim that another genus, *Trichomitus* (Fig. 3), is also present, but, for reasons given in this paper, it is impossible to say whether or not *Trichomitus* is present. If it is, it reacted in every way as *Trichomonas* did, and has the same relation to its host and fellow protozoa as *Trichomonas*.

Two methods, starvation and oxygenation, for removing the protozoa are given. By using each method separately and by a combination of the two it was possible to get five different combinations of the protozoa, without injuring the termites in the least. By starving for 6 days, *Trichonympha* was removed entirely; by starving for 8 days, *Leidyopsis* was removed completely; by oxygenating for 25 hours, *Trichomonas* was entirely removed; by oxygenating for 24 hours and starving for 6 days, *Trichomonas* and *Trichonympha* were removed; by oxygenating for 24 hours and starving for 8 days, *Trichomonas*, *Trichonympha* and *Leidyopsis* were removed; by oxygenating for 72 hours, all protozoa were removed. From this we get one group of termites with no protozoa in them and five groups with a different combination of protozoa in each group as follows: (1) *Leidyopsis*, *Trichomonas*, *Streblomastix;* (2) *Trichomonas*, *Streblomastix;* (3) *Streblomastix;* (4) *Trichonympha*, *Leidyopsis*, *Streblomastix;* (5) *Leidyopsis*, *Streblomastix;* and (6) no protozoa. By feeding the normal diet of wood to each of these groups of termites it was possible to work out the relation of each protozoan genus to its host and to its neighbors or fellow protozoa.

When group (1), which contained *Leidyopsis*, *Trichomonas* and *Streblomastix*, was fed wood, *Leidyopsis* multiplied rapidly, increased greatly in number and was able in 20-30 days to fill the vacant space made in the host's intestine when *Trichonympha* was removed. This group is able to live indefinitely.

When group (2), which contained *Trichomonas* and *Streblomastix*, was fed wood, *Trichomonas*, like *Leidyopsis* in group (1), multiplied rapidly and increased greatly in number for about 30 days, but was never able to fill the intestine with protozoa, that is to say, *Trichomonas* was never able to entirely take the place of *Trichonympha* and *Leidyopsis* in volume. Most of the termites of this group were able to live 70–80 days and some of them longer, although very few, if any, were able to live indefinitely. *Trichomonas*, then, is of some value to its host as a symbiont. It can keep its host alive 40–50 days longer than the host would be able to live without it. If *Streblomastix*, which is certainly not of any value to its host and may have to be supported by *Trichomonas* in this group, were not present, *Trichomonas* might be of more value to its host.

When group (3), which contained *Streblomastix*, was fed wood, death resulted within three to four weeks, the same time it occurs when all protozoa are removed. *Streblomastix* did not multiply at all; on the contrary, it gradually diminished in number. This protozoön, then, is not a symbiont. It may either receive its nourishment from its host or from the other protozoa directly, probably the latter.

When group (4), which contained *Trichonympha*, *Leidyopsis* and *Streblomastix*, was fed wood, all individuals were able to live indefinitely. The removal of *Trichomonas* did not seem to affect the symbiosis at all. *Trichonympha* perhaps took the place of *Trichomonas* very quickly.

When group (5), which contained *Leidyopsis* and *Streblomastix*, was fed wood, *Leidyopsis* multiplied and increased in numbers just as it did in group (1), perhaps taking the place of *Trichomonas*—though this could not actually be seen—just as it did that of *Trichonympha*. These termites were able to live indefinitely. *Leidyopsis*, even though in nature its ratio to *Trichonympha* is approximately 1 : 1000, can take the place of *Trichonympha* under experimental conditions both in number and in ability to keep its host alive indefinitely. In nature, however, *Trichonympha* must be the chief symbiont, for it is so much more numerous than its closest neighbor, *Leidyopsis*.

When group (6), which contained no protozoa, was fed wood, death resulted within three to four weeks.

The results of these experiments, as regards the symbiotic relationship between termites and their intestinal protozoa, are in accord with those obtained by the incubation method (Cleveland, '24*a*). The symbiosis between these insects and their intestinal flagellates has now been clearly demonstrated by three entirely different methods, namely, incubation, starvation, and oxygenation.

ADDENDA.

Since this paper went to press sometime ago several additional observations of interest have been made.

Thirteen colonies of *Termopsis nevadensis* and six colonies of *Termopsis angusticollis* have been obtained. The protozoa in all of these are the same.

By dry fixation and staining with Wright's stain one and three minutes respectively it has been possible to demonstrate beyond question four anterior flagella on *Trichomonas*. No organisms with three anterior flagella, the number *Trichomitus* is said to possess, have been observed. Hence it is quite probable that *Trichomitus* does not occur in *Termopsis*. Another interesting result was obtained by this method of fixation and staining: It was discovered that *Streblomastix* has only four flagella, instead of six, the number figured by Kofoid and Swezy ('19). The flagella stand out as big red lines which may be counted almost as easily as so many red pencils. Perhaps this method of fixing and staining will prove valuable in determining the flagella number of other protozoa.

By oxygenating *Termopsis* at a pressure of 1.5 atmospheres for 7 hours it has been possible to remove both *Trichomonas* and *Streblomastix* from many, though never all, hosts without seriously effecting *Trichonympha* and *Leidyopsis*. When termites oxygenated in this way are starved for six days, *Trichonympha* disappears entirely, thus making it possible to obtain termites with only *Leidyopsis*. This gives two more protozoal combinations, in addition to the five already obtained, *viz.* termites with *Trichonympha* and *Leidyopsis*, and termites with *Leidyopsis*. It now seems desirable to tabulate all the protozoal combinations; show how they were obtained, and how, if at all, they effect their hosts.

TABLE I.

RESULTS OF VARIOUS METHODS WHICH HAVE BEEN EMPLOYED IN REMOVING ONE OR MORE GENERA OF PROTOZOA FROM THE LARGE PACIFIC COAST TERMITE, *Termopsis nevadensis* Hagen.

Every host in nature always harbors each genus. − = absent, *i.e.*, treatment killed all protozoa of this genus and + = present, *i.e.*, treatment had no effect.

Methods of Treatment.	The Protozoa.				Result of Treatment on Host.
	Trichonympha.	*Leidyopsis.*	*Trichomonas.*	*Streblomastix.*	
1. Starvation for 6 days	−	+	+	+	Lives indefinitely.
2. Starvation for 8 days.	−	−	+	+	Lives about 10 weeks.
3. Oxygenation for 24 hours at 1 atm. . . .	+	+	−	+	Lives indefinitely.
4. Oxygenation for 24 hrs. at 1 atm. Starvation for 6 days.	−	+	−	+	Lives indefinitely.
5. Oxygenation for 24 hrs. at 1 atm. Starvation for 8 days.	−	−	−	+	Lives 3–4 weeks.
6. Oxygenation for 7 hrs. at 1.5 atms. . .	+	+	−	−	Lives indefinitely.
7. Oxygenation for 7 hrs. at 1.5 atms. Starvation for 6 days	−	+	−	−	Lives indefinitely.
8–13. Oxygenation, 1 atm., 72 hrs., 1.5, 9 hrs., 2, 5 hrs., 2.5, 2 hrs., 3, 1 hr. and 5 min., 3.5 40 min.	−	−	−	−	Lives 3–4 weeks.

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EXPLANATION OF PLATE I.

The Protozoa of *Termopsis*. Figs. 1–4 after Kofoid and Swezy; Figs. 5, 6 original.

FIG. 1. *Trichonympha campanula*. × 300.

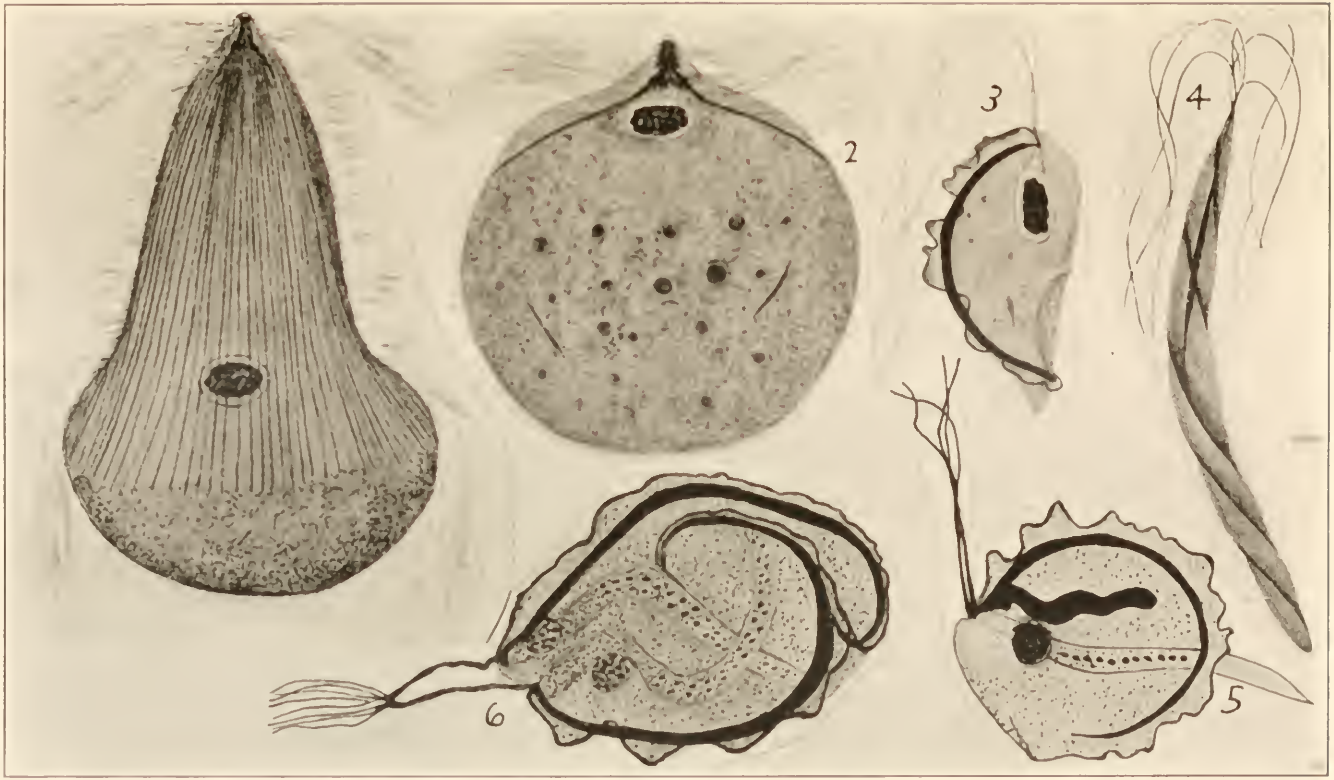
FIG. 2. *Leidyopsis sphaerica*. × 300.

FIG. 3. *Trichomitus termitidis* which may be a synonymn of *Trichomonas termopsidis* (Figs. 5, 6). × 625.

FIG. 4. *Streblomastix strix*. × 2500.

FIG. 5. *Trichomonas termopsidis* fixed in weak Flemming's fluid. Note parabasal body which is not present in Fig. 6 because this organism was fixed in Schaudinn's fluid which nearly always makes the parabasal invisible. × 1440.

FIG. 6. *Trichomonas termopsidis* dividing form fixed in Schaudinn's fluid. Paradesmose has just disappeared. Note doubling of nuclei, axostyles, anterior flagella, undulating membrane, and chromatic basal rod of undulating membrane. Parabasal body does not appear because of fixation in Schaudinn's fluid. × 1440.



L. R. CLEVELAND.

SOME PHYSIOLOGICAL DISTINCTIONS BETWEEN FRESHWATER AND MARINE ORGANISMS.

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I.

Aquatic organisms are in general distinctly divided into those species inhabiting fresh water and those inhabiting sea water. Yet this division is evidently not a phylogenetic one, since closely related species live in either (Quinton, '04). Only in a few species are the same individuals capable of passing from one medium to the other. The transition zones of brackish water are relatively small in extent and very variable, still there are a few organisms which thrive only in such diluted sea water.

Particularly the distribution of fishes between fresh water and sea water has excited the interest of biologists. Paul Bert tried to find why fish died when taken from their usual medium and placed in the other ('71, '73). For marine animals he ('83) reached one important conclusion, namely, that the toxic effect was not always due to the dilution of the substances dissolved in sea water. This conclusion has been thoroughly confirmed by the subsequent observations of Loeb ('03) and others.

Practically no further analysis of this problem could be made without careful quantitative studies. Such were undertaken by Sumner ('06) upon marine teleost fishes. He found significant changes in the water content and in the salt content of the animals during their immersion in diluted sea water. Thus the deleterious effect seemed to be a loss of essential body constituents, because the animals were unable to regulate favorably their integumental permeability under the new circumstances.

Meanwhile a new line of attack came to light with the discovery of salt antagonisms by Ringer ('82). Loeb ('00) found that marine organisms were readily affected by the disturbances of the balance between salts, but were almost independent of osmotic pressure changes. Thus ('03), the marine crustacean *Gammarus* endured dilution of the seawater medium just as well if the diluting fluid was distilled water as if it was a sugar solution isotonic with sea water.

It is important to realize that various groups of animals differ greatly in their ability to control the interchange of substances between body fluids and external environment (Semper, '80). In all metazoa the covering epithelial layer of the body carries on an active regulation of the interchanges, so that the outer medium affects the more vital tissues only after it has with surprising slowness changed the composition of the internal medium (Adolph and Adolph, '25). Thus in all these animals there are two lines of defense, two regulating surfaces which must be penetrated. To illustrate several grades of this ability to control the interchanges at the body surface, we may cite the mammals, in which it is well known that the skin is impermeable at all times; the aquatic amphibia, in which the permeability is controlled in such a way that significant chemical interchanges between body and environment are strongly opposed; and the annelid worms, in which the interchanges are still less selected. In the teleost fishes essential interchanges are limited to the gill surfaces (Sumner, '06). In elasmobranch fishes the entire skin is partially permeable, while in invertebrates the body fluid often interchanges freely with the medium.

We have attempted to compare the vital resistance to the penetration of chemical agents in freshwater animals and marine animals. For this purpose we have chosen those animals whose entire body surface is normally permeable in some degree, namely, small invertebrates. In them the surface mass ratio is sufficiently large that effects of the chemical environment quickly manifest themselves.

II.

An excellent measure of the amount of integumentary regulation performed by organisms is the rate of change of body weight when the organisms are transferred from their usual medium to a solution of different chemical composition (Adolph and Adolph, '25). Thus, when freshwater animals are immersed in salt solutions there is a rather sudden loss of water, so that the body volume quickly reaches a new lower level. Seawater animals placed in diluted sea water gain in volume, even though the dilution be carried out with an isotonic solution of a non-electrolyte. Several species of invertebrates, both freshwater and marine, have been compared in this manner. The flatworm *Dendrocœlum* and the earthworm *Lumbricus*, both of which inhabit fresh water, reach the new volume level in about 5 hours. The marine annelid *Phascolosoma*, when placed in sea water diluted to half its normal concentration, attained the new volume level in less than 2 hours. Evidently *Phascolosoma* adjusts to the new environment more rapidly than the freshwater species; it has less resistance to the penetration of water through its surface.

We wished to know whether dissolved substances likewise penetrated at different rates through the body coverings of these two classes of animals. After the first adjustment to the immersion in salt solution by loss of water, freshwater animals remained at the new level of volume for several days, and when replaced in fresh water recovered two thirds of the original loss. *Phascolosoma*, however, in diluted sea water lost slowly a considerable amount of the dissolved substance of the body fluids, so that when finally returned to normal sea water very little water passed from the body compared to that originally gained.

A still larger number of animals we have compared with respect to their resistance to solutions of salts and other substances. A list of these species, and the highest concentrations of certain solutes which they can just survive for a considerable length of time, are given in Table I. An important conclusion can immediately be drawn from these data; namely, that for freshwater organisms the osmotic pressure of the medium usually limits survival, while for marine organisms a great range of concentrations can be resisted. Thus, marine Gammarus will live indefinitely if transferred to sea water diluted with distilled water up to 0.5 per cent. (0.005 M), or concentrated by the addition of salts up to 160 per cent. (1.56 M, corrected for ionization). Freshwater *Gammarus*, on the other hand, are usually killed by immersion in any solution of a concentration equivalent to 0.35 M. Gradual dilution or concentration of the medium does not appreciably extend these limits.

The general significance of the conclusions from all the above experiments we interpret to be that both dissolved substances

TABLE I.

MAXIMUM SURVIVAL CONCENTRATION OR PERCENTAGE OF VARIOUS DISSOLVED SUBSTANCES WHICH WERE ENDURED BY 3 FRESHWATER SPECIES AND 3 MARINE SPECIES AT 22° C. FOR THE ARBITRARY TIMES SPECIFIED.

Figures in parenthesis are molar concentrations, corrected for ionization.

Solutions in Distilled Water.	Sea Water.	Urea, M.	Glycerol, M.	Glucose, M.	Sucrose, M.	NaCl, M.	KCl, M.	$CaCl_2$, M.	Ringer,[1] M.	$MgCl_2$, M.
Gammarus fasciatus (Crustacean) 5 hrs.	100% (0.97)	0.35	0.35	0.38	0.30	0.20 (0.35)	0.04 (0.07)	0.03 (0.08)	0.26 (0.43)	0.14 (0.35)
Phagocata gracilis (Flatworm) 24 hrs.	35% (0.33)	0.32	0.32	0.32	0.23	0.16 (0.28)	0.04 (0.07)	0.04 (0.11)	0.15 (0.26)	0.10 (0.25)
Paramecium caudatum (Protozoan) 1 hr.	17% (0.16)	0.32	0.25	0.20	0.20	0.11 (0.20)	0.07 (0.13)	0.05 (0.14)	0.11 (0.20)	0.12 (0.30)
Isotonic Solutions Added to Sea Water.	Distilled Water, %.	0.88 M Urea, %.	0.88 M Glycerol, %.	0.88 M Glucose, %.	0.88 M Sucrose, %.	0.54 M NaCl, %.	0.54 M KCl, %.	0.30 M $CaCl_2$, %.		
Gammarus locusta (Crustacean) 5 hrs.	99½	70	99½	65	85	80	5	21		
Procerodes wheatlandi (Flatworm) 24 hrs.	98	70	97	90	95	50	4	40		
Copepod (Crustacean) 1 hr.	60	60	60	50	60	60	3	15		

[1] Unbuffered Ringer's solution was made by mixing 97 volumes of 0.54 M NaCl, 2 volumes of 0.54 M KCl, and 1 volume of 0.30 M $CaCl_2$. This was taken to be 0.54 M.

and water penetrate the tissues of marine animals faster than they pass through the covering layers of freshwater animals. But in both cases the presence of salts in abnormal proportions in the medium renders the permeability of the integument high.

The greater permeability to salts and water in marine organisms accords with the ordinary conditions of existence for these animals. Marine animals are constantly bathed in a medium which is physiologically as suitable in inorganic composition as any internal one (Fredericq, '22). Freshwater animals, on the other hand, cannot afford to lose dissolved substances from their bodies nor to allow the entrance of water into their bodies up to the point where osmotic equilibrium would result.

Osmotic pressure changes *per se* are evidently not particularly deleterious to the vitality of internal tissues, providing the integument does not attempt to regulate against them. In marine organisms the freer penetration of solutes allows the internal medium to keep pace with the outer medium as regards composition, and so long as the salt balance is preserved, no essential functions are inhibited.

Morphologists have often attributed the chemical resistance of freshwater organisms to their possession of an outer cuticulum. Perhaps the resistance referred to is the resistance to the penetration of protein-precipitating agents, and in this a proteinaceous cuticulum probably assists. But under ordinary conditions the cuticulum is by no means either impermeable or semipermeable. Rather the living integument is responsible for the maintenance of restricted or selective permeability.

III.

A second significant characteristic of freshwater animals appears to be that their body fluids have a lower osmotic pressure than those of marine organisms.

Whether there is a relation between toxicity and tonicity we have attempted to investigate by measuring the survival of plasmolyzed *Spirogyra* filaments. With a variety of plasmolysing agents it was found that the toxic concentration was almost exactly the lowest one which produced distinct permanent plasmolysis. By gradually increasing the concentration of the medium, both toxicity and plasmolysis were prevented. Now, in all the freshwater animals studied, toxic effects followed immersion in a concentration of about 0.35 M non-electrolyte solutions, or the equivalent concentration of electrolytes. Fredericq ('98) and Botazzi ('08) have shown that the tissue fluids of freshwater animals always have osmotic pressures less than half of that of sea water ($\Delta = 0.8°$ C.), and usually have only one eighth to one fourth of that of sea water; while marine animals have body fluids which are exactly isotonic with sea water. We can thus probably regard the maximum survival concentration for freshwater animals as a measure of the osmotic pressure of their body fluids. All the freshwater animals studied evidently had, therefore, internal osmotic pressures equivalent to 0.20 to 0.35 M.

The acclimatization of marine organisms to changed osmotic conditions contrasts to that for freshwater organisms. In several instances marine animals placed in glass-distilled water died, yet most of them survived a mixture of 98 or 99 per cent. of distilled water with only 2 or 1 per cent. of sea water. In other words, most marine animals are able to live after abrupt change to almost pure fresh water, providing that the remaining salts are present in physiological proportions. Gradual dilution of the sea water over several days did not materially help marine animals to endure pure water, though complete acclimatization has been secured over long periods of time by other observers (Beudant, '16, Plateau, '71, Semper, '80).

For freshwater organisms, gradual increase in the concentration of the medium did not greatly increase the maximum survival concentration. It is evident from this that plasmolytic effects are not the important ones in producing this toxicity. In diluted sea water all the freshwater animals studied except *Gammarus* were killed in concentrations less than half of that of the seawater at Woods Hole ($\Delta = 1.81°$ C.), and acclimatization never increased this toxic limit up to half of the concentration of the sea water. Similar acclimatizations to specifically poisonous substances have been demonstrated by Davenport and Neal ('96) and numerous other investigators, so that it seems doubly certain that the ultimate toxic effect is not plasmolytic. Moreover, the toxic effect cannot be attributed to sudden volume or concentration changes such as are brought about by diffusion. Certain balanced solutions such as sea water can be resisted in spite of their high concentration by freshwater *Gammarus*; there is no interference with the regulatory activity of the integument. At this point we approach the problem of the conditions for the survival of the internal tissues, which are evidently very different from the conditions which the external medium may impose when an integument is interposed.

IV.

It appears, therefore, that freshwater organisms are strongly contrasted to marine organisms with respect to their ability to adjust to changes in their chemical environment. Marine organisms in general maintain a higher degree of interchange of inorganic materials with their surroundings. Freshwater organisms, on the other hand, have a more restricted and selective interchange, and thus dissolved materials are kept inside their bodies and water is kept outside. In protecting themselves from their environment through the retention of salts, freshwater organisms have laid themselves open, paradoxically, to osmotic disturbances of the integument. Marine organisms, by allowing the environing medium to serve as the physiological fluid, are able to endure a much greater change in the environing medium, than are freshwater organisms with their greater regulation of the internal medium. This greater change is one of concentration only, however, for both groups of animals are equally susceptible to variations in the proportions of salts in the medium. This condition in the freshwater animals is entirely due to the peculiar and variable type of permeability found in the integuments of this group, whereby they normally maintain a concentration difference between outside and inside fluids, as has been pointed out previously (Quinton, '04, Adolph and Adolph, '25). This activity of the integument serves excellently in the normal medium, but adds a factor of susceptibility.

Existence in the freshwater medium is accompanied by the possession by all freshwater organisms of relatively dilute tissue fluids. Apparently it is the integumentary activity which is upset by increased concentrations of the medium. Its upset, in turn, produces rapid changes of a deleterious nature in the composition of the body fluids. Marine organisms, on the other hand, have body fluids which are in complete chemical equilibrium with their environments (Quinton, '04). Thus it turns out that while the essential physiological constitution of the tissues of the two groups of animals does not differ, the limiting factors for the proper functioning of their regulatory integuments differ vitally.

It seems probable that the transition from one type of integumental permeability to the other type occurs automatically during the long process of acclimatization which accompanies the transfer of an organism from one aquatic medium to the other; that both types are inherent in all integuments.

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ENDOCRINE GLANDS AND BILATERAL SYMMETRY: OBSERVATIONS UPON FORELIMB ERUPTION IN FROG LARVÆ UNDER TREATMENT WITH THYROID AND THYMUS EXTRACTS.

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INTRODUCTION.

A conspicuous feature of thyroid-accelerated metamorphosis in frog larvæ is the eruption of the forelimbs. Before these become visible externally they are located beneath the skin in the gill chamber. For some years the writer has been aware of the fact that the left forelimb is protruded before the right forelimb, sometimes by as much as several days. This condition was recorded in a previous paper (Jordan and Speidel, '23) and has probably also been noted by other workers in the field of amphibian metamorphosis. In half-grown bullfrog and green frog tadpoles no exceptions to this were seen; *i.e.*, no case appeared of precedent right forelimb eruption in thyroid-treated tadpoles. It was, therefore, of some interest to find in a jar of seven thymus-treated tadpoles one animal in which the right forelimb appeared two weeks before the left. Furthermore, in two other animals in this lot the amount of skin degeneration in the right forelimb region was definitely farther advanced than that on the left side, a condition indicating probable prior right forelimb eruption. Death ensued, however, before the appearance of either forelimb. Two of the other four animals in this jar put out the left forelimb first, and in one the amount of skin degeneration on the left side plainly foreshadowed the prior eruption of the limb of that side. The remaining animal put out both limbs over night. In this jar of seven thymus-treated animals, therefore, the ratio of "right-handed" to "left-handed" animals is 1 : 1.[1]

The question suggests itself as to whether the endocrine secretions represented by the thyroid and thymus extracts actually affect in a differential manner the bilateral symmetry of the developing animals. Further experiments were set under way in an attempt to analyze the factors controlling variation in forelimb eruption.

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[1] The terms "right-handed" and "left-handed" are used to denote merely prior right forelimb eruption or prior left forelimb eruption, respectively.

There is a difference of opinion as to the condition in normal frog larvæ. Barfurth ('87) in Europe finds in the case of *Rana fusca* that 80 per cent. put out the right forelimb first, the left almost always following in from two to eight hours. On the other hand, Gudernatsch ('14), using *Rana temporaria* and *Rana esculenta*, states that he has always found the reverse to be the case; *i.e.*, in about 80 per cent. the left forelimb erupts first.[2] Dickerson ('20) observes that in normal bullfrog and green frog metamorphosis the left forelimb is usually put out first. My own observations upon the normal tadpoles of *Rana sylvatica*, *Rana clamata*, *Rana catesbeiana*, and *Hyla crucifer* lead me to agree with Gudernatsch and Dickerson that in a definite majority of cases the left limb is the first to erupt.

MATERIAL, EXPERIMENTS AND OBSERVATIONS.

The material used includes about 800 tadpoles of *Hyla crucifer*, *Rana catesbeiana*, *Rana clamata*, and *Rana sylvatica*, and a few of *Rana cantabrigiensis* and *Rana pipiens*. The bullfrog and green frog tadpoles were collected at Charlottesville, Virginia, the others at Woods Hole, Massachusetts. Untreated animals were usually kept in aquaria containing pond water and weed. Administration of endocrine extract was accomplished by placing some of the extract in the water with the animals. Thyroid and thymus desiccated extracts were used.

The accompanying table indicates the relative frequency of left or right forelimb eruption, as it occurs under normal conditions, under thyroid treatment, and under thymus treatment.

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[2] Gudernatsch makes this statement in a footnote referring to his experiment with thyroid-accelerated metamorphosis. It is probable, therefore, that he included his observations on thyroid-treated animals with those on normal animals in regard to forelimb eruption, not realizing that thyroid treatment affects forelimb eruption. Thyroid administration, as shown by this paper, markedly favors the prior eruption of the left forelimb. His percentage, therefore, is not correct, but is too high in favor of lefthandedness.

TABLE I.

In this table is given for each species of tadpole the number of individuals observed with prior left forelimb eruption as compared with the number of individuals observed with prior right forelimb eruption, under normal conditions, under thyroid treatment and under thymus treatment.[3]

	Normal-untreated.		Thyroid-treated.		Thymus-treated.	
	Prior Left.	Prior Right.	Prior Left.	Prior Right.	Prior Left.	Prior Right.
Hyla crucifer	43 (72%)	17 (28%)	25 (100%)	0	17 (50%)	17 (50%)
Rana sylvatica	42 (65%)	22 (35%)	19 (100%)	0		
Rana catesbeiana and *R. clamata*	3	0	85 (100%)	0	3 (50%)	3 (50%)
Rana cantabrigiensis			4 (100%)	0		
Rana pipiens			20 immature tadpoles, all died before either forelimb erupted			
Total, all species	88 (70%)	39 (30%)	133 (100%)	0	20 (50%)	20 (50%)

Under normal conditions a definite majority of tadpoles puts out the left forelimb first; in *Hyla* 72 per cent., in *Rana sylvatica* 65 per cent. The few observations upon untreated bullfrog and green frog tadpoles in the three-limb condition support Dickerson's observation that the left limb usually erupts first. Under thyroid treatment the left forelimb is invariably protruded first in all species studied, if those putting out forelimbs during the first forty hours of the treatment are excluded. Under thymus treatment apparently a 1 : 1 ratio is indicated. The experiments with *Hyla* afford the best comparative figures and may be taken as typical.

With thyroid treatment of half-grown tadpoles 100 per cent. put out the left forelimb first. A special experiment was tried in which 75 *Rana sylvatica* tadpoles were subjected to thyroid treatment, many of these at the time being on the verge of putting out the forelimbs. Among those animals putting out forelimbs within the first forty hours, seven righthanded ones were seen; thereafter all were lefthanded. It may be concluded that in animals protruding the forelimbs within this time, an original bias toward righthandedness may not be changed. In a similar experiment with 25 *Hyla* tadpoles that were also fairly close to the time of forelimb eruption, one righthanded animal occurred during the first 24 hours; after that all were lefthanded. For this reason, in Table I. all animals putting out forelimbs during the first two days are omitted from the reckoning, since the original normal bias may not have been sufficiently influenced by the endocrine extract.

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[3] For reasons explained in the text all animals putting out forelimbs within the first two days of thyroid or thymus treatment are discarded, and do not figure in the table.

How long it takes for the thymus extract to affect an original bias is not known. Since it is probably not so powerful as the thyroid it may be that more than the first two days' results should be discarded. If the first two days are discarded the ratio is 17 : 17 as given in the table. If the results of the first three to seven days are discarded the ratio shifts progressively to favor righthandedness. Only a much larger number of animals under observation would give a trustworthy ratio. The figures given, however, are in all probability enough to show that the normal ratio has been affected and shifted in the direction of righthandedness.

Interpretation and Discussion.

These observations leave no doubt that the bilateral symmetry of the developing frog tadpole, as indicated by the relative time of forelimb eruption, is influenced by thyroid extract; possibly also by thymus extract. The action of the thyroid extract will first be discussed. The blood carries the active thyroid principle to all parts of the body. It is inconceivable that the thyroid autacoid should have one effect on the tissues on the left side and another effect on exactly similar tissues on the right side. Therefore, the effect of the thyroid in changing the normal symmetrical development must be due to some original fundamental asymmetry of the body pattern.

The following findings are pertinent: The tadpole is conspicuously asymmetrical in respect to its respiratory apparatus. A spiracle, or outlet from the gill chamber, is present on the left side only (Fig. 1). This outlet drains both left and right gill chambers, these being connected by a canal across the mid-line. The forelimb is present in the gill chamber, its degree of development depending upon the general developmental state of the tadpole. Both in normal and thyroid-induced metamorphosis the left limb is pushed through the spiracle, a variable amount of preliminary skin degeneration occurring. The right forelimb erupts through the skin only after the latter has undergone a certain amount of degeneration. This degeneration starts in the vicinity of the elbow, and the elbow is usually protruded first. Movements of the forelimb finally enable it to break completely through. In young thyroid-treated tadpoles, however, the forelimbs are little developed so that the elbow is not prominent at the surface. In these cases the hand or whole arm appears as a tiny white stump after the skin degeneration has proceeded far enough.[4]

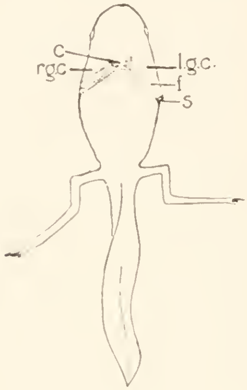

FIG. 1.

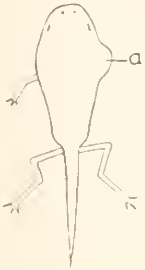

FIG. 2.

FIG. 1. Ventral view of a tadpole of the tree frog (*Hyla crucifer*) showing well developed forelimbs (*f*) still imprisoned beneath the skin in the gill chambers (*r.g.c.* and *l.g.c.*). The position of the sinistral spiracle (*s*) is shown, which drains both gill chambers; also the canal (*c*) across the mid-line which connects right and left gill chambers. The dotted line below the forelimbs indicates the position of the partition separating the gill chambers from the abdominal cavity.

FIG. 2. Dorsal view of a thyroid-treated tadpole (*Hyla crucifer*) in typical three-limb stage after the eruption of the left forelimb. Collection and retention of air (*a*) in the right gill chamber causes bulging out of the skin and interferes with forelimb eruption on that side. Excluding the first two days, prior left forelimb eruption occurs invariably.

The administration of thyroid extract to a half-grown tadpole obviously upsets the respiratory mechanism. More air is taken in. But apparently the animal is not yet very well fitted for utilizing it properly. Small bubbles of air collect in the gill chambers and are not expelled, or expelled with difficulty. This is especially true of the right gill chamber which has no outlet except through the left spiracle (and, of course, the mouth). This proves to be the deciding factor. The left gill chamber is usually drained well enough except in very immature tadpoles, so that skin degeneration and forelimb eruption on that side are not interfered with. On the right side, however, the retention of air in the chamber causes bulging out of the skin (Fig. 2), and interferes to a greater or less degree with the normal eruption on that side, thus bringing about the typical prior left forelimb eruption. A tadpole in this stage does not present a normal posture when at rest, but floats with the right side somewhat elevated owing to the air in the right gill chamber. The difference between a thyroid-treated animal in this condition and either normal or thymus-treated animals may be seen by comparing Fig. 2 with Figs. 3 and 4. Occasionally, in very young and immature tadpoles no forelimbs erupt with thyroid treatment, the animals dying in the two-limb stage. In these, bubbles of air may be seen in both gill chambers. The pulmonary

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[4] While this paper was in press, Helff ('24), at the Washington meeting of the American Society of Zoölogists, reported a series of experiments which show clearly that the opercular skin autolysis preceding forelimb eruption is brought about by substances given off by the adjacent atrophying gills.

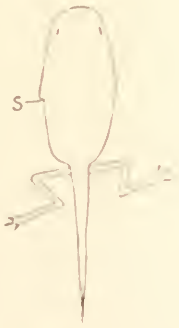

FIG. 3.

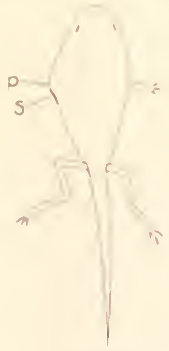

FIG. 4.

FIG. 3. Dorsal view of a normal tadpole (*Hyla crucifer*) before the eruption of either forelimb. The location of the spiracle (*s*) on the left side only, leads to a majority (70 per cent.) of prior left forelimb eruptions.

FIG. 4. Dorsal view of a thymus-treated tadpole (*Hyla crucifer*) showing prior right forelimb eruption. Either right or left forelimb may erupt first. In the region of the spiracle (*s*) may be seen an angular projection (*p*) caused by the pressure of the imprisoned left forelimb.

development of the animal is not adequate to the demands imposed upon it by thyroid treatment and death results. Coupled with this respiratory disturbance is also the condition of anemia already pointed out elsewhere (Jordan and Speidel, '23). The older the tadpole the more probable it is that both forelimbs will erupt. The pulmonary apparatus is presumably better developed, and the forelimbs are large enough so that limb movements aid both in expelling the air from the gill chamber and in breaking through the skin. In thyroid-treated animals that are near the time for metamorphosis both forelimbs are put out with little trouble, the sinistral location of the spiracle becoming of less importance.

In a recent paper by Swingle ('23) one figure is given to show the effect of iodo-tryosine administration in accelerating metamorphosis in pituitaryless *Rana sylvatica* tadpoles. After sixteen days of treatment the specimen illustrated has one forelimb, that one being a right forelimb. Swingle does not state when this particular right limb appeared but does say that two right forelimbs broke through as early as the eighth day, the average, however, being about twenty days. This is an interesting observation in comparison with my results after thyroid treatment; *i.e.*, 100 per cent. prior left forelimb eruption after the first two days. It seems to mean, either that iodo-tryosine does not affect the respiratory apparatus in the same way as does thyroid extract, or that the pituitary gland plays a rôle also in influencing symmetrical development.

It now remains to discuss the normal condition and the thymus-treated condition. Three factors are considered of chief importance in determining forelimb eruption: (1) sinistral location of the spiracle; (2) relative degree of skin degeneration over the forelimb region on the two sides of the body; (3) relative size and strength of the forelimb. In partly grown thyroid-treated animals the first factor is by far the most important, as has been shown. In mature untreated tadpoles, however, this factor does not remain the all-important one. The forelimbs are now so large that the left one cannot be pushed through the spiracle without a fair amount of previous skin degeneration. Simultaneous skin degeneration occurs on both sides. The size, strength and activity of the imprisoned forelimb now becomes of much importance. In about 30 per cent. of cases the right forelimb succeeds in overcoming the handicap of having no spiracle to come through, and breaks through by main strength before the left. In the other 70 per cent. the left limb aided by the sinistral spiracle comes through first. A majority in favor of lefthandedness is about what should be expected.

Mention should be made again of Barfurth's results. In normal *Rana fusca* tadpoles he finds that 80 per cent. put out the right forelimb first. Presumably these are like all other frog tadpoles in having sinistral spiracles although Barfurth does not refer to this feature. He believes the prevailing righthandedness of this species is accounted for by two factors: (1) earlier and greater degeneration of the skin in the right forelimb region, and (2) greater size and strength of the right limb. As these results are directly opposed to the observations of Gudernatsch, Dickerson and myself on five species of frog tadpoles, it can only be supposed that there is a species difference, and that the factors mentioned by Barfurth are strong enough to bring about a majority of righthanders in this particular species. It would be of interest to see whether in this species also the uniform lefthanded condition could be produced by thyroid treatment.

The explanation of the results after thymus treatment is somewhat more difficult and uncertain. It is probable that a shift toward righthandedness is here indicated. The ratio may be 1 : 1, although on account of the small number of animals observed in the three-limb stage, this is by no means a certainty. At any rate, the lessening of the lefthanded majority means that the asymmetrical position of the spiracle becomes of much less importance as a factor in determining the first forelimb to appear.

Desiccated thymus extract is a food rich in nutritive value, and therefore favorable to growth. Gudernatsch ('14) noted its growth-promoting effect upon tadpoles and ascribed it to the endocrine secretion of the thymus. Uhlenhuth ('17) though he combats Gudernatsch's idea as to the growth effect being due to an endocrine secretion contained in the thymus extract, states that it is a very rich and nutritious food and therefore quite favorable to growth. The writer has also observed that it is particularly favorable to limb growth. In one batch of partly-grown thymus-treated green frog tadpoles the small hind limbs became quite red and vascular and grew rapidly, almost reminding one of the effect of thyroid extract. The writer became suspicious of the thymus extract used and had it analyzed for the presence of iodine.[5] The analysis gave negative results. The later history of the tadpoles showed that only growth in size of the larval structures was being stimulated, and not differentiation. There was no acceleration in skin degeneration of the forelimb region, except that caused secondarily by pressure of the growing forelimb. There was likewise no reduction in the tail, but on the contrary growth. With thymus treatment limb growth appears to proceed relatively faster than the general process of body differentiation. As a result, the forelimbs enclosed beneath the skin reach a comparative size and strength, such that they become the important factor in determining the time of eruption. With increasing limb size the spiracle becomes less important since the forelimb cannot be pushed through it without complementary skin degeneration. Since size of forelimb and amount of skin degeneration on the two sides are about equal in the species under observation a more equal ratio of righthandedness to lefthandedness results.

The writer does not wish here to enter the controversy as to whether or not the thymus extract has a specific endocrine content. Its effect upon symmetry in forelimb eruption in tadpoles seems to be best explained on the grounds given above; *i.e.*, its unquestioned value as a highly nutritious and therefore growth-promoting food. It is not necessary to assume a specific endocrine effect. This much, however, may be added. Thymus gland is largely lymphoid tissue. In the light of Carrel's work showing the growth-promoting effect of leucocytic secretions or "trephones" ('24), and the confirmatory observations of Jordan and Speidel ('23) on lymphocytes in rapidly growing regions in tadpole metamorphosis, it would seem probable that growth-promoting substances (trephones) of lymphocyte origin would be present in thymus extract.

In conclusion, it may be pointed out that these observations and their interpretation, though of little importance in themselves, suggest the possibility of the following principle operating in any vertebrate animal in process of development: A change in the normal balance of thyroid secretion may lead to a change in the symmetrical development. Stockard ('23) has emphasized the general importance of thyroid secretion in the development of man and mammals and its part in determining the production of definite types. These results on forelimb eruption in tadpoles indicate that thyroid secretion may be of some importance also in influencing symmetrical development. A vertebrate animal, though designated as bilaterally symmetrical, is, of course, asymmetrical in many respects, *e.g.*, the visceral pattern, much of the vascular system, etc. Given an original asymmetrical condition it is possible that the thyroid may exert its effect upon the two sides of the body in a differential way.

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[5] The analysis was made by Mr. T. F. Otto, of the University of Virginia Medical School.

SUMMARY.

The bilaterally symmetrical development of the frog larva is affected in a definite way by experimental hyperthyroidism. Normally in tadpole metamorphosis the left forelimb erupts first in about 70 per cent. of cases. With thyroid-accelerated metamorphosis of half-grown tadpoles practically 100 per cent. will put out the left forelimb first. Of 133 thyroid-treated animals of this kind every one protruded the left forelimb first. In the case of full-grown tadpoles already near the time of forelimb eruption, thyroid treatment may be followed during the first two days by some prior right forelimb eruptions; thereafter prior left forelimb eruption obtains. In other words, an original bias of an animal toward prior right forelimb eruption may not be changed by thyroid administration within two days.

This effect of the thyroid on symmetrical development is explicable in terms of the original asymmetrical pattern of the respiratory apparatus (*i. e.*, sinistrally located spiracle) coupled with the close anatomical relation of the forelimb to this apparatus.

Thymus treatment brings about a larger percentage of prior right forelimb eruptions, thus reducing somewhat the normal majority in favor of prior left forelimb eruption.

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CELL SIZE AND METABOLIC ACTIVITY IN AMPHIBIA.

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I. INTRODUCTION.

The fact that the mass of a body increases as the cube of the linear dimension, while the surface increases as the square, has long been recognized as of importance in biology. Leuckart (1852) uses it to explain the necessity for increased surface as an organism becomes larger, a necessity which is met in animals by inpushings and the develompent of a distributing system; in plants by outgrowths. Herbert Spencer (1873) says: "Why has the individual a growth limit? . . . In similarly shaped bodies, the masses vary as the cube of the dimensions, whereas the strengths vary as the square of the dimensions." He applies this idea to an individual whose height doubles in a given growth period; the mass has been multiplied by eight, but the strengths of muscles and bones, being proportional to their cross section, are multiplied by only four. The absorbing surface is also multiplied by four, while the mass to be nourished by the material absorbed is multiplied by eight. It is only a step farther to apply the same idea to cell size. The writer was unable to learn who first did this.

Since the absorbing surface of a cell increases only twice as fast as the radius, while the mass to be nourished by absorbed material increases four times as fast, it follows that there is a definite size limit to cell growth; and further it is evident that the absolute size attainable by any given cell is inversely proportional to its rate of consuming the material absorbed. In other words, a sluggish cell may absorb the relatively small amount of material needed for its activity through a smaller surface than a more active cell, with its greater requirements of material; or the sluggish cell could grow to a larger size than the active one and still get sufficient material through its surface. If this is true, then one may reasonably expect a sluggish animal to have larger cells, while a more active animal would require smaller cells.

The research described in the present paper was started with the idea of obtaining some experimental data which might indicate whether the assumption stated above is true, and therefore whether the size of cells might be of fundamental importance in the activities of an animal. The Amphibia were chosen for experimental material because they have quite large cells which can be measured with less error than smaller cells, and because the Amphibia are known to vary quite widely in both cell size and activity.

Gulliver (1875) published measurements on the blood corpuscles of 650 species of Vertebrata; including 3 Cyclostomata, 11 Elasmobranchii, 75 Pisces, 17 Amphibia, 38 Reptilia, 265 Aves, and 241 Mammalia. Perusal of his figures with a consideration of the relative degree of activity of the various animals indicates a general agreement with that to be expected if cell size does vary inversely with activity, but there are numerous exceptions. Most of these exceptions can be explained on the basis of the size of the animal, for Gulliver points out that, within a limited group, the size of the red blood corpuscles increases with increasing weight of the members of the different species considered. That activity may be of importance in connection with cell size is indicated by the fact that, among the Mammalia, the smallest corpuscles are found in the deer family, the largest in the elephant, porpoise, anteater, and sloth and near the average size among the Carnivora. Among the Cheiroptera the fruit-eating bats have distinctly larger corpuscles than the insect eaters. Among the birds the largest corpuscles are found among the Cursores, and the smallest among the insect-eating passeriform birds. Among the reptiles the Chelonia have distinctly larger corpuscles than the Sauria. The corpuscles are much larger in the Caudata than in the Salientia, and larger in the Elasmobranchii than in the Pisces.

Other workers who have given measurements of red blood cells are Weckler (1863), Malassez (1872), Formad (1888), Wormley (1888), and Forrest (1913). Forrest and Malassez also made counts. These run in inverse ratio to the size, although there are exceptions. A comparison of the measurements of amphibian corpuscles by different workers will be found later. Reichert and Brown (1909) review the work which has been done on red blood cell size and state that attempts to correlate the size of these cells with the rapidity of the animals' movements are founded on insufficient or erroneous data.

Hartmann (1919*a*) shows that the chloroplasts in developing *Elodea* leaves are smaller and more numerous in plants grown at higher temperatures, as contrasted with the larger and less numerous chloroplasts in plants grown at lower temperatures. Since the metabolism of the leaves is certainly speeded up with increased temperature, this observation falls well in line with the idea that a high rate of activity is associated with small size.

Chambers (1908) shows that there is considerable variation in the size of the eggs of *Rana esculenta* and *R. temporaria*, that the larger eggs develop a little more rapidly than the smaller ones, and that there is a much higher percentage of mortality among small than among large eggs, especially when grown at higher temperatures. He shows that the size of the cells in the frog varies with the size of the egg from which the frog developed; and that eggs allowed to develop at higher temperatures invariably yield smaller frogs with smaller cells than those developed at lower temperatures (size taken at time of metamorphosis or earlier). Tadpoles in crowded cultures are smaller than those with more room, but this does not affect the size of the cells. Morgan (1904) worked on dwarf frog eggs which had only about half the volume of the normal eggs, and showed that the cells in the developing dwarf embryos tend to remain smaller than normal. Berezowski (1910) worked on the size of the intestinal cells of the white mouse during development, and shows that these cells become larger as the animal grows.

Krogh (1916) gives comparative tables on basal metabolism as it has been worked out by various workers on different animals. These figures indicate that there is a general agreement between activity as measured by basal metabolism, and cell size as measured on the red blood cells by Gulliver (1875), in inverse ratio. Figures given by different workers on metabolism vary quite widely, and this is true to an extreme degree of Amphibia. For instance Regnault and Reiset (quoted in Morat and Doyon, 1900) found that 0.063 mg. of CO_2 was eliminated per gram of frog per hour, while Krogh (1916) gives a figure which corresponds to 0.3686 mg. of CO_2 per gram per hour.

II. Material and Methods.

As many different species of Amphibia were used as it was possible to obtain. Activity was measured in terms of carbon dioxide output. This was measured by fixing the gas as a precipitate of barium carbonate in a barium hydroxide solution. In detail the method consisted in sucking air by means of a filter pump through one 8-inch tube of concentrated potassium hydroxide, two 8-inch tubes of soda lime and a gas washing bottle containing strong barium hydroxide. This series was to remove the carbon dioxide from the atmospheric air. The stream of air then passed into a respiration chamber containing the animal. Even when air breathing animals were used some water was always placed in the bottom of this chamber to keep the skin of the animal moist. From the respiration chamber the air current passed through two or three gas-washing bottles containing a carefully measured amount of standardized barium hydroxide. Special care was taken to see that the air was broken up into fine bubbles as it passed through these bottles. To accomplish this the end of the inlet tube was drawn out into two fine points. The bulb type of bubbler was found unstaisfactory because it was too easily broken in the numerous manipulations incident to making a long series of determinations; and because a finer stream of bubbles could be obtained by the method described. Suction tubing was used in making connections, and special precautions were taken to avoid leaks.

Standard solutions were made by preparing a stock solution of N/10 oxalic acid by weight, N/10 barium hydroxide standardized against this, and N/10 hydrochloric acid standardized against the barium hydroxide. Phenolphthalein was used as an indicator in preparing the standards and in the actual determinations. A preliminary aëration was run with the animal in the respiration jar, but without the collecting bottles, for one hour. The collecting bottles were then placed in the series, and aëration carried on for a measured length of time, 8–24 hours. At the end of this aëration the collecting jars were removed and the excess of hydroxide titrated immediately by means of N/10 hydrochloric acid. The amount of hydroxide used by the carbon dioxide was thus obtained by difference, and the amount of carbon dioxide collected computed as CO_2 per gram body weight per hour. All determinations were made on starving animals and at room temperature, which varied between 20 and 23° C.

Truog (1915) describes a method of determining carbon dioxide by passing air through a tower containing barium hydroxide and glass beads. This method would be more accurate than the one used here, but the method was unknown to the writer at the time when the experiments described in this paper were undertaken. It is felt that the method used here yields results of comparative value, which is all that is needed. Truog shows that the barium carbonate present with the hydroxide does not hinder accurate titration, and that the barium hydroxide method of determining carbon dioxide is very accurate.

Red blood corpuscles were used for measuring cell size. Most of the animals used were those on which carbon dioxide determinations had already been made. The animal was killed either by pithing or with chloroform, and blood taken either from the heart by means of a syringe or from the tail vein. Thin smears were made on slides, dried in the air and stained with Wright's stain. In many cases blood counts on both red and white corpuscles were made, and tissues fixed in Bouin's fluid for section later in order that other cells might be measured. The blood counts and tissue cell measurements are not included in the present paper. The latter agree reasonably well with the results given for red blood cells, while blood counts are found to vary widely with the physiological condition of the animal. Of course in general animals with larger cells have smaller numbers.

Red blood cells were measured by means of a scale which was so constructed that each division on the scale corresponds to one micron in the oil immersion field (B. & L. 1.9 mm. obj.) with 10x ocular and 160 mm. tube length, when the scale is placed on the table beside the microscope and viewed through a camera lucida with mirror set at an angle of 45° and the arm length set at 103 mm. With the aid of this scale the dimensions of red blood cells were measured, 50 cells being measured from each slide, and from one to five slides being used for each animal studied. From the average length and width thus obtained the surface of the average corpuscle for each animal was computed, assuming no thickness, from the formula $\pi LW/2$ where L is the maximum and W the minimum diameter. This formula follows from the formula for a regular ellipse, πab, where a and b are the long and short radii. The surface is used as the significant figure rather than the length and width because all corpuscles are not the same shape, length and width have different ratios, and therefore the dimensions do not give a direct index of size.

III. Results for the Different Species of Amphibia.

Table I. gives the results for the various species used. Where a space is left blank, no data were obtained on the particular point concerned. For instance it will be noticed that sex is not given in a number of cases, and in the same cases usually no blood cell measurements are given. In these cases the animal died and it was not considered safe to make blood cell measurements on such animals in which post mortem changes had had time to occur. Therefore they were not autopsied at all, and thus no data were obtained on sex. In several species it was impossible to obtain samples for carbon dioxide determinations, although one or several had already been used for blood smears.

A short description of the material and results for each species is given below. The species are taken in the same order as in the table; that of red blood cell size.

Amphiuma means (Gordon).—This species has the largest corpuscles known for any amphibian. Three adult specimens and one young were obtained from New Orleans. Measurements of carbon dioxide output were made at intervals over a period of two weeks. The animals showed evidence by their

TABLE I.

CELL SIZE AND CARBON DIOXIDE PRODUCTION FOR THE DIFFERENT INDIVIDUALS USED IN EACH SPECIES STUDIED.

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Cm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Amphiuma means.							
F 1	large			50	62.8	35.42	3.494
F 2	41.4	0.0920	3	150	60.9	34.8	3.329
3	1,243	0.02136	3	50	62.18	36.8	3.594
4	1,270	0.02146	3	50	62.06	37.42	3,648
5	1,500	0.02125	2	50	61.0	39.04	3.741
Necturus maculosus.							
F 2	154	0.0420	5	250	53.78	30.8	2,602
M 3	194	0.0413	5	250	57.45	30.62	2,763
F 4	126	0.05285	16	250	54.568	27.496	2.357
M 5	130	0.0548	4	250	50.152	26.012	2,049
M 6	115	0.0532	10	150	52.386	26.46	2,177
M 7	126	0.05576	13	25	55.52	29.24	2.550
F 8	57.6	0.0922	12	250	51.144	30.984	2.489
M 9	55.7	0.1069	13				
M 10	86	0.0883	1	50	54.44	30.54	2,612
11	47	0.1399	2				
F 12	75	0.0915	1				
F 13	93.5	0.0842	2				
M 14	92	0.0753	1	50	54.04	25.7	2,187
15	74	0.1051	1				
F 16	44.3	0.1208	3	50	50.38	31.66	2,505
F 17	64	0.1192	2	50	53.72	25.28	2,133
18	194	0.0599	1				
Cryptobranchus alleganiensis.							
1				50	41.12	23.16	1,496
Diemyctylus viridescens.							
1				50	29.64	17.76	827
Rana catesbiana							
F 1	455	0.0629	4	250	25.2	13.032	516
F 2	604	0.0527	3	250	27.324	13.444	578
F 3	714	0.05257	4	50	25.58	12.86	517
F 4	510	0.0929	5	50	25.62	16.18	651
M 5	361	0.0671	3	250	26.836	13.176	555
6	503	0.0584	3				
M 7	388	0.0704	3	50	25.38	14.14	564
8	492	0.0627	3				
M 12	507	0.0876	3	50	24.48	13.84	532
F 13	411	0.0699	3	100	26.07	16.82	702
14	361	0.0787	3				
F 15	628	0.0843	3	50	23.86	14.16	531

Table I.—*Continued.*

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Rana clamitans.							
1......				530	22.072	12.498	433
F 2......	19	0.1794	4	250	23.236	12.22	446
M 3......	7.1	0.1359	4	100	23.64	11.03	409
Rana pipiens.							
F 1......	43.4	0.1386	9	100	19.53	12.56	385
M 2......	25.4	0.1422	17	150	20.37	11.32	362
F 3......	45.8	0.1768	7	250	21.078	14.192	470
M 4......	27.0	0.2026	5	250	22.96	13.644	492
M 5......	30.5	0.1762	13	50	19.86	13.78	421
M 6......	33.6	0.1394	10	50	21.24	12.96	432
F 7......	59.0	0.1795	3				
F 8......	43.5	0.1931	6	50	18.46	13.42	389
F 12......	54.7	0.1145	2				
F 13......	52.6	0.1314	1				
M 14......	32.7	0.1103	1				
F 15......	40.8	0.1765	5				
M 16......	28.9	0.2002	7				
M 17......	14.4	0.2037	1				
Rana palustris.							
1......				100	20.47	13.94	448
F 2......	5.7	0.2742	5	200	20.11	12.705	401
M 3......	3.6	0.2918	8	250	19.12	12.16	365
F 4......	3.7	0.2531	9	250	20.384	12.152	389
F 5......	3.9	0.1710	2	100	21.58	12.44	422
6–17....	36	0.1603	2				
18......				50	20.7	13.96	454
M 19......				150	19.13	12.47	375
Hyla pickeringii.							
1......				50	19.44	11.2	342
Acris gryllus.							
1......				50	17.82	11.18	313
2......				50	17.7	10.6	295

TABLE I.—*Continued.*

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Chorophilus nigritus.							
1......				50	17.96	10.04	283
Bufo americanus.							
F 2......	11	0.1831	5	250	16.044	9.684	244
3......	1.9	0.2069	1				

feces of having eaten recently. The baby specimen was killed and blood smears made immediately at the termination of the carbon dioxide output measurements; the adults were bled from the tail vein after six weeks.

Necturus maculosus (Rafinesque).—All specimens except the first two used were collected from Lake Mendota at Madison. They were kept in cold running water without food until used. From the time of the first determination on an individual until it was killed or died it was kept at room temperature. The first two specimens were received from dealers, and their source is unknown. In this species starvation was carried on over a long period of time, and its effect on metabolism studied. Individuals of widely varying weights were used and the effect of weight on metabolism noted. The results of these experiments will be described later.

Cryptobranchus alleganiensis (Daudin).—The single specimen used was a laboratory specimen of unknown source from which blood smears were made. No individuals were available when needed for metabolism tests on account of the cold weather.

Diemyctylus viridescens (Rafinesque).—One specimen was used for blood smears. It was collected in New York state during the summer, and blood smears were made in the fall. Probably the animal had not eaten in the meantime.

Rana catesbiana (Shaw).—Twelve specimens were obtained from New Orleans. Specimen No. 1 had had the lower jaw broken at some previous time, and it had healed in such a manner that eating was impossible and the animal was in an extreme state of starvation. The other specimens showed signs of having recently eaten, and were well fed. They were kept at a temperature of about 12° C. when not being used for carbon dioxide output tests. Attention is called to specimen No. 4, which shows an unusually high carbon dioxide output. It is not included in the general average for the species in Table VIII. The detailed record for this animal follows: 2/10—0.0653, 2/29—0.0639, 3/20—0.1208, 4/18—0.1037, 4/20—0.1110 (dates of determinations and carbon dioxide output figures). No explanation is found for this strange behavior; starting with approximately a normal carbon dioxide output value, jumping to twice the normal, and remaining there. That the phenomenon is not due to an acute infection is indicated by the fact that the animal appeared normal on autopsy a month after the final test.

Rana clamitans (Latreille).—Two specimens were collected near Madison and kept at room temperature until used three months later. They had no food during this time. Blood smears were made from No. 1 immediately after it was collected. The low value for carbon dioxide production with the small animal is probably due to the extreme emaciation of this specimen. Neither cell size nor activity results differ essentially from those of the following species.

Rana pipiens (Shreber).—The specimens used were from several shipments from supply houses. Some of these animals were starved at room temperature for a long time, and the effects of lack of food on metabolism were noted. No appreciable decrease in metabolism resulted until the animal had reached an extreme state of starvation. No. 2 lost 47 per cent. of his body weight before any marked drop in carbon dioxide output per gram of body weight was obtained. Several specimens died of disease. The results of the study of metabolism during the course of the disease will be described later.

Rana palustris (Le Conte).—The specimens used were collected near Madison in the late fall. They were kept at room temperature and used for carbon dioxide output tests over a period of two months. At the end of this time they were all very weak from starvation. Nos. 1, 2, and 3 were killed while still in good condition. No. 5 and Nos. 6–17 were tested when near death from starvation. No. 18 was starved, while No. 19 was used for blood smears within a few days after being collected.

Hyla pickeringii (Holbrook).—One specimen was collected and blood smears made before the carbon dioxide tests had been started. None were available while tests on metabolism were being made.

Acris gryllus (Le Conte).—Two specimens were collected from the field and blood smears made. Time was lacking to make carbon dioxide output tests.

Chorophilus nigritus (Le Conte).—See note on *Hyla pickeringii*.

Bufo americanus (Le Conte).—Two specimens were collected and starved at room temperature three months before using. No. 3 died of starvation after only one test for carbon dioxide had been made, and blood could not be obtained for smears.

IV. Controls.

A. Cell Size.

1. Blood corpuscles may shrink as smears dry. However the same methods were used on all specimens, so that the results should be comparable. That there is a certain amount of differential shrinking due to differences in thickness of smear is suggested by comparing measurements from different slides made from the same individual. For instance the five slides of blood from Rana clamitans No. 2 gave the following series of averages of 50 corpuscles from each slide: 23.98 x 13.02, 22.92 x 11.66, 23.32 x 12.08, 23.1 x 12.32 and 22.86 x 12.02. Some of this variation may have been caused by differences in the corpuscles which happened to be measured, but most of it was probably due to the nature of the smear. With the slide which averaged 23.98 x 13.02 the first 25 corpuscles measured 23.84 x 13.12, while the last 25 measured 24.12 x 12.92. The method used is that of Gulliver (1875) except for the fact that he does not appear to have stained his smears. Georgopolus (1906) states that dry preparations are unreliable because the size of the cells is likely to vary with the thickness of the film, and states his preference for the wet method. This consists in placing a small drop of fresh blood on a clean slide and quickly placing on a cover. The corpuscles are then measured immediately. This method has been found useless for Amphibia because the corpuscles are distorted by the

treatment. For the smaller mammalian corpuscles it is an excellent method. The one attempt which was made to use the wet method gave sizes which checked fairly well with measurements from dried smears. It was found to be difficult to use an oil immersion objective on a wet preparation, and it was difficult to find corpuscles which were not distorted.

For convenience of reference for the reader, and to show how the present measurements check with those given by other workers, Table II. has been prepared to show the sizes of amphibian corpuscles according to the measurements of other workers. The writer's measurements are not included here because they are mostly on different species, the table would therefore be much longer, and the measurements are found elsewhere in this paper.

Table II.

Measurements on Amphibian Red Blood Corpuscles as Made by Different Workers.

Species.	Gulliver.	Weckler.	Wormley.	Forrest.	Morat and Doyon.
Amphiuma tridactylum (means).....	69.9 x41.3		70.9x40.9		
Proteus angineus...	63.5 x34.94	58.2x33.7			58.0x35.0
Siren lacertina.....	60.47x33.42				
Cryptobranchus japonicus.........	56.45x31.75				
Cryptobranchus alleganiensis.......	45.11x25.4				
Siredon humboldtii..	44.8 x25.4				
Lissotriton punctatus	31.75x19.84				
Salamandra.......				37.8x23.8	
Triton bibronii.....	29.95x19.84	29.3x19.5		29.3x19.5	29.3x19.5
Triton cristatus....	29.95x19.84				
Rana.............		22.3x15.7	23.3x14.1	22.3x15.7	22.3x15.7
Rana esculenta.....	25.4 x17.58				
Rana temporaria...	22.92x13.95				
Bufo.............		30.2x18.2		21.8x15.9	
Bufo vulgaris......	24.35x12.7				
Bufo clamita.......	19.05x13.4				

2. It is possible that some of the animals studied by the writer show abnormally small corpuscles on account of extreme starvation. *Necturus* No. 6 and *Rana pipiens* No. 2 are cases with extreme starvation and small cells. However, *Necturus* Nos. 7 and 8 and *Rana pipiens* No. 5 also underwent extreme starvation,

and show relatively large cells. *Rana palustris* No. 19 was collected from the field and blood smears made immediately, yet this specimen shows the smallest corpuscles measured for this species. On the other hand *Rana palustris* No. 18 shows the largest corpuscles of the species, and was used when in an extreme state of starvation. There seems to be no correlation between cell size and degree of starvation, as judged by the size of the red blood cells.

B. Carbon Dioxide Determination.

1. The apparatus used has two inherent defects. These are not considered to be of sufficient importance to affect the results for the relatively large amounts of carbon dioxide measured. They are, first that rubber tubing was used for all connections, and second that soft glass bottles were used for collecting jars. Rubber tubing has been shown to have a selective absorption for carbon dioxide, but this should not be important considering the short lengths of tubing used, the rapidity of the air stream, and the relatively large amounts of carbon dioxide collected.

2. To learn whether the traps to remove carbon dioxide from the air before it entered the respiration chamber were taking out all the gas, a gas washing bottle containing a carefully measured amount of standard barium hydroxide was placed between the traps and the respiration chambers. 22 hours of rapid aëration yielded 5.06 mg. of CO_2, or 0.23 mg. per hour.

3. To learn whether some of the expired CO_2 was getting by the collecting jars, a barium hydroxide bottle as in the previous case was placed between the collecting jars and the pump. A rapid stream of air passing through two respiration jars and two sets of collecting jars (in parallel; one for *Necturus*, one for *Rana pipiens*), was sucked through this jar for 26 hours. 28.6 mg. of CO_2 were collected, or 1.1 mg. per hour. Subtracting from this figure the amount introduced into the jars with the inhaled air, it appears that 0.87 mg. per hour of CO_2 was being lost from the system. The animals in the jars weighed 93 grams, so that the loss is 0.00936 mg. per hour per gram weight of the animals. This is about an 8 per cent. loss for *Necturus* and about 6 per cent. for the frog. For the larger animals an additional collecting jar was used. This of course tended to keep down the loss.

4. Leaks were practically eliminated. It was possible to develop a strong negative pressure in the jars, close all valves, and allow the apparatus to stand for several hours with no appreciable diminution of pressure.

5. The negative pressure in the respiration jars averaged about 2 cm. of mercury. This factor was practically constant for the entire course of the experiments.

6. Measurements of the rate of flow with the air current moving at as near the average rate as was possible to judge yielded two minutes for each liter of air. This stream was divided between a jar of four liters capacity (used for *Necturus*, *Rana catesbiana* and *Amphiuma*) and one of one liter capacity (used for the smaller animals). Tests on each jar separately showed that the air in the large jar was being changed every eleven minutes, while that in the small jar was changed every five minutes. This should be sufficient speed in each case to keep the atmosphere around the animal relatively free of carbon dioxide. Attempts to cut down the rate through the small jar to more nearly equal that of the larger jar were unsuccessful because it was found that, with too slow a rate, the holes in the bubblers in the gas collecting jars became clogged with precipitate of barium carbonate and the aëration stopped.

7. Determinations of the dissolved carbon dioxide in the water surrounding the animal at the end of the preliminary aëration and again at the end of the final aëration yielded approximately the same figure in each case. For instance, while a carbon dioxide test was being made on a bull frog, no titratable CO_2 was found in the water used in the jar, 5.5 mg. CO_2 per 100 cc. were present in the water at the end of the preliminary aëration, and the same figure at the end of the final aëration.

8. Several blanks were run with the regular amount of water in the respiration jars, but without animals, and one with a completely empty jar. The results of these trials are shown in Table III.

For all the above tests 250 cc. of N/10 barium hydroxide were measured into two gas-washing bottles, and the bottles filled to capacity (300 cc.) with distilled water. This same procedure was followed in filling the jars for the regular carbon dioxide output tests. For numbers 7 and 8 solutions were used which were not quite standardized. Therefore No. 8 is of value only in comparison with No. 7. Subtracting the two it is found that the 14 hours of aëration resulted in the accumulation of 11 mg. of CO_2, or 0.785 mg. per hour. Comparing this with No. 6 it is seen that 1.275 mg. per hour resulted from the presence of water in the respiration jar in the aëration numbered 6. The source of this CO_2 is the dissolved bicarbonate of the water, which gradually liberates carbon dioxide when CO_2 free air is bubbled through it. This is not a factor when an animal is in the jar, as is shown by the determination recorded above of the amount of dissolved CO_2 in the water surrounding a respiring animal; sufficient to prevent liberation of the gas from the bicarbonate. Trials Nos. 1, 2, 3 and 5 indicate that this combined CO_2 comes out quite slowly. No. 4 represents the degree of accuracy which can be expected from the titration. This figure represents 0.8 cc. of N/10 barium hydroxide in 250 cc.; and thus is an error of 0.32 per cent.

TABLE III.

BLANK TESTS ON CARBON DIOXIDE.

Trial No.	Hrs. of Aëration.	Mg. CO_2 Collected.	Mg. CO_2 per Hour.	Remarks.
1	5	5.02	1.012	Small jar. One liter capacity.
2	9	5.72	0.635	Small jar.
3	14	8.36	0.597	Small jar.
4	0	1.76	—	Treated as for aeration, but titrated immediately.
5	27	11.98	0.444	Small jar.
6	24	49.5	2.06	Large jar. Four liters cap.
7	14 (24)	18.48	0.486	No water. Large jar. 14 hrs.' aëration plus 24 hrs. standing.
8	(38)	7.48	0.197	Parallel with No. 7. No aëration; standing.

It appears, then, that the carbon dioxide collected in the blank aërations is from three sources: (1) that due to the liberation of the bound CO_2 of the water, (2) that caused by residual CO_2 in the atmosphere of the respiration chamber at the end of the preliminary aëration, and (3) that resulting from CO_2 getting through the air washing system before the air enters the respiration chamber. Of these only the last is of importance in producing error, the others being eliminated by the presence of a respiring animal in the jar. This factor has already been shown to be overcompensated by the loss resulting from incomplete absorption of the carbon dioxide by the barium hydroxide. Inasmuch as the first two factors are important, they would tend to neutralize this loss. It is therefore concluded that the method is sufficiently accurate to allow for making comparative carbon dioxide output determinations on the animals used.

Indirect evidence that the factors discussed above are not important in producing error is obtained by comparing the results obtained on different animals. Errors caused by the passing through of excess carbon dioxide would tend to increase the apparent result for small animals more than for larger ones, while for small animals the error resulting from the loss of carbon dioxide that was not absorbed would tend to be minimized. With these ideas in mind, if one looks at the results for *Rana palustris* (Table I.) it is apparent that Nos. 6–17 have within the limits of variation the same carbon dioxide output result as No. 5, although the latter has twelve times the chance of being thrown off by the errors as have the former. Again *Bufo americanus* shows only slightly higher results for a 1.9 gm. individual as for an 11 gram one, and *Rana clamitans* shows a lower result for the lighter animal.

V. Complicating Factors.

A. Cell Size.

1. If the figures presented in this paper are compared with those obtained by other workers, it will be found that they run decidedly low. Measurements are presented for the same species only in the case of *Amphiuma* and *Cryptobranchus*, but the indications are in the same direction for the frogs and toads, in which different species have been used here than those used by other workers. There are two possible causes for this difference; first that the writer has obtained shrinkage of the corpuscles, as previously pointed out, and second that the measuring technique used is faulty. Several attempts have been made to check the latter point. The scale used has been repeatedly compared with a Zeiss stage micrometer, and found to be accurate. By means of an ocular filar micrometer the widths of the different 10 micron divisions of the Zeiss stage micrometer have been measured.

TABLE IV.

VARIATION IN RED BLOOD CELL SIZE IN DIFFERENT SPECIES OF AMPHIBIA.

Number of corpuscles measured corresponding to each size.

Size Micra.	*Amph.*		*Nec.*		*Crypt.*		Size Micra	*Diem.*		*R. Cat.*		*R. Clam.*		*R. pip.*		*R. pal.*		*Hyla.*		*Acris.*		*Cero.*		*Bufo.*	
	L.	W.	L.	W.	L.	W.		L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
83	1						34			1															
77	2						33	1		3															
76	1						32	7		6															
74	1						31	11		16															
73	2						30	10		38															
72	3						29	9		109		3													
71	6						28	5		120		5													
70	5		2				27	4		149		22		4											
69	7						26	3		230		40		4		1									
68	8		5				25	3		214		77		30		4									
67	18		7				24			97		140		82		16		1							
66	17		7				23			62		155		111		57									
65	24		15				22			37		121		150		98		3							
64	14		12				21			14	1	104		143		142		8							
63	37		16				20		5	7	1	86		162		305		8		7		7			
62	32		33				19		10	1	7	45		122		202		18		18		12		3	
61	27		34				18		16		33	24		63		128		10		37		14		37	
60	32		62				17		10		51	8		16	3	51		2		22		9		53	
59	19		52				16		5		46	1	2	4	13	6	1			14		5		67	
58	21		85				15		3		148		29		132		21			2		3		65	
57	13		99				14		1		292		105		296		112							18	
56	15		98				13				314		186		233		323		3		3			6	
55	16		139				12				130		231		124		379		15		28		2	1	1
54	7		107				11				44		148		58		144		22		32		8		30
53	3		111				10				24		78		33		20		9		30		31		121
52	2		167				9				2		1		11				1		8		8		85
51	5		122				8																1		13

TABLE IV.—*Continued.*

Size Micra.	*Amph.*		*Nec.*		*Crypt.*		Size Micra.	*Diem.*		*R. Cat.*		*R. Clam.*		*R. pip.*		*R. pal.*		*Hyla.*		*Acris.*		*Coro.*		*Bufo.*	
	L.	W.	L.	W.	L.	W.		L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
50	5		164																						
49			95																						
48	1		74																						
47			74																						
46			49		1																				
45	1	1	31		4																				
44	1	5	9		3																				
43		4	9		8																				
42		7	2		8																				
41		13	1		5																				
40		28	1	1	7																				
39		21	1	6	4																				
38		37		9	2																				
37		31		21	3																				
36		52		33	3																				
35		47		57	1																				
34		28		61																					
33		28		110		1																			
32		20		131																					
31		14		151																					
30		7		176																					
29		4		161																					
28		2		149																					
27				138																					
26				123		3																			
25				138		3																			
24				95		10																			
23				63		8																			
22				36		10																			
21				20		7																			
20				6		3																			
19				1		1																			
Totals	350		1700		50		Totals	50		1100		830		900		1000		50		100		50		250	

It is found that these divisions vary in width from 5.8 per cent. below the mean to 5.6 per cent. above it. With such wide variations between the different divisions of the micrometer, the question naturally arises as to whether the entire micrometer may not be inaccurate. As a third check on the method the dimensions of corpuscles as measured by means of the scale were compared with the dimensions of the same corpuscles as measured by means of the filar micrometer. It was found that the scale is less accurate for individual corpuscles, because it is impossible to measure with it to an accuracy of less than one micron, but the average of a series of measurements by the two methods gave closely parallel results.

2. In the Amphibia the size of individual red blood cells in the same animal varies so widely that averages only partly represent the peculiarities of the different species. In many cases the range of variation in size is just as characteristic as the average size. For this reason Table IV. has been prepared to show the range of size variation in each species. In many cases

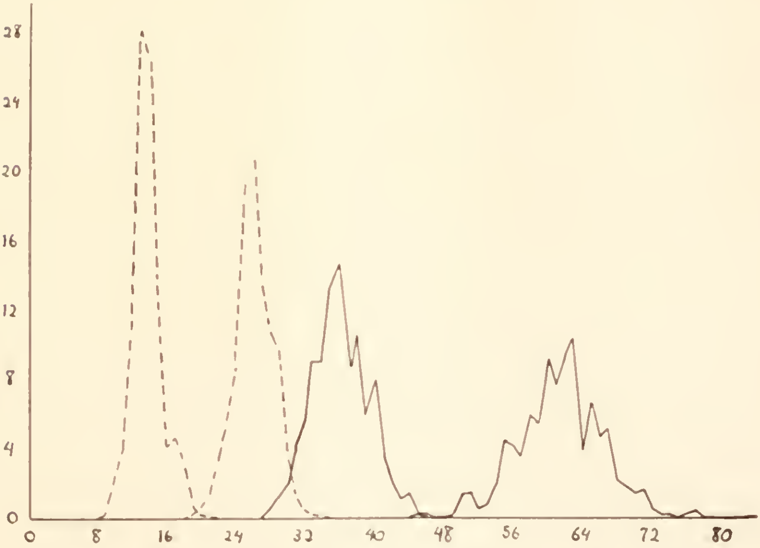

CHART 1*a*. Variation in red blood cell size for *Amphiuma* (solid line) and *Rana catesbiana* (broken line). Abscissa, dimensions in micra; ordinate, percentage of total corpuscles measured. Two curves are given for each species, one representing the long diameters of corpuscles, and the other the short diameters.

there are either two maxima or a sustained maximum. This results from the fact that the maximum for different individuals of the same species varies. It is just this variation between individuals which complicates comparative results on cell size, especially between closely related species. It is not associated with the metabolic activity of the animal, sex, or any other factor which can at present be indicated. Reference to Chart I will aid in understanding the variations here discussed.

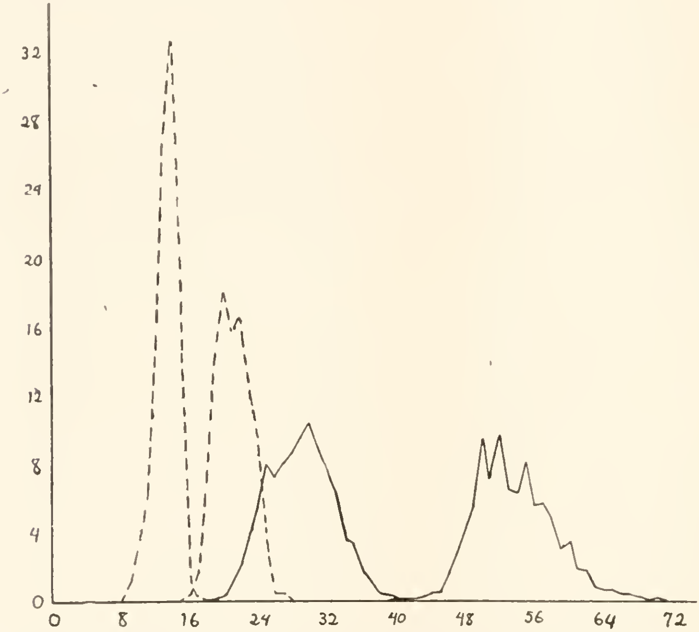

CHART 1*b*. Variation in red blood cell size for *Necturus* (solid line) and *Rana pipiens* (broken line). Explanation as for Chart 1*a*.

B. Carbon Dioxide Production.

1. The weight of the animal is the primary factor which complicates the results on carbon dioxide production. The general fact is that the carbon dioxide output as measured by unit weight increases as the weight decreases. Reference to the results for *Necturus* will emphasize this fact. For convenience these are listed in Table V., the individuals being taken in order of their weight.

TABLE V.

Necturi ARRANGED ACCORDING TO WEIGHT TO SHOW VARIATION OF CARBON DIOXIDE PRODUCTION WITH VARIATION IN WEIGHT.

No.	Weight, Grams.	CO_2, Mg.	No.	Weight, Grams.	CO_2, Mg.	No.	Weight, Grams.	CO_2, Mg.
3	194	0.0413	6	115	0.0532	17	64	0.1192
18	194	0.0599	13	93.5	0.0842	8	57.6	0.0922
2	154	0.0420	14	92	0.0753	9	55.7	0.1069
5	130	0.0548	10	86	0.0883	11	47	0.1399
4	126	0.0528	12	75	0.0915	16	44.3	0.1208
7	126	0.0557	15	74	0.1051			

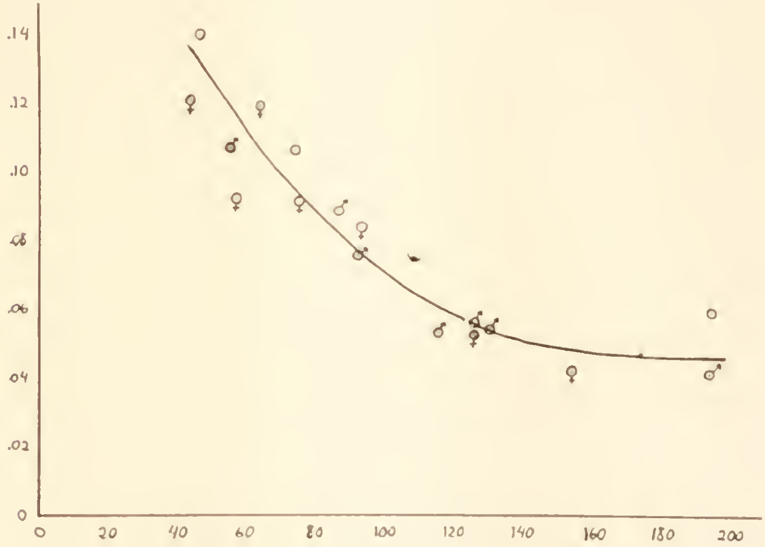

CHART 2. Influence of weight on carbon dioxide output in *Necturus*. Abscissa, weight in grams; ordinate, CO_2 in milligrams per gram of body weight per hour, ♀ = females, ♂ = males, ⊙ = sex not determined.

The general trend is evident from Table V., but exceptions are also evident. Nos. 18, 13, 15, 17, and 11 are higher than would be expected from their weight, while Nos. 2, 6, 14, and 8 are lower. The high group contains only animals on which one or two carbon dioxide output tests could be made before the animal died. The cause of death in all these animals was probably starvation, there being no pathological symptoms as far as could be determined. A more detailed discussion of starvation as a complicating factor will be found below. It is more difficult to understand the meaning of the abnormally low carbon dioxide production. All the individuals in the low group except No. 14 were used for a number of trials; 14 died after 105 days of starvation when only one trial had been made. Nos. 2 and 8 are females; 6 and 14 males. Females usually run lower than males (see below) but this explains only two of the cases. The detailed record of No. 6 shows a low initial period, a fluctuating intermediate period, and a very high final one as starvation progressed. The final period was characterized by a rapid loss of weight.

In the other species studied the same inverse ratio between body weight and metabolism is evident. In *Amphiuma* the result is strikingly higher for the smallest animal, and unusually constant for the three larger specimens. In the frogs the same trend may be observed, but it is not as much in evidence, probably because of the preponderance of other complicating factors.

These observations suggest that weight is not a thoroughly satisfactory basis for computing basal metabolism in these animals. The ideal basis on which to make such computations would be mass of respiring tissue in the body. In Amphibia this mass would be less in proportion to the total body weight in *Salientia* than in *Caudata*, due to the greater mass of bone in the former. This proportion would also be smaller in large animals than in small individuals of the same species, because of the increased ossification and connective tissue in the former. In man it has been found that the body surface is the more reliable criterion, and elaborate formulæ have been worked out for computing this surface from the weight and height. In Amphibia such formulæ would be useless, on account of the great variation in shape which is found between the various species. It appears that the most hopeful method of eliminating weight variations in comparing different species as to carbon dioxide output is to choose animals from each species which have approximately equal weights. In the final section of this paper an attempt is made to do this.

2. Starvation is a factor leading to important variations in the results, especially with *Necturus*. The rate of carbon dioxide elimination increases as starvation progresses. This fact has already been indicated by the observations on Necturi with

abnormally high carbon dioxide output. Table VI. and Chart 3 illustrate this variation. The two specimens chosen were both starved for a long period, and determinations were made throughout the period.

Both these animals show unmistakably the upward trend of the production of carbon dioxide with increased length of starvation, even beyond that which can be accounted for on the basis of decreasing weight during starvation. The writer was led to make the computations on the basis of standard weight because a preliminary examination of the results had suggested that the actual increase observed was largely or entirely due to this factor. These results mean, then, that the reduced amount of living tissue resulting from starvation actually produces a greater absolute amount of carbon dioxide than the greater amount of living tissue present at the beginning of starvation.

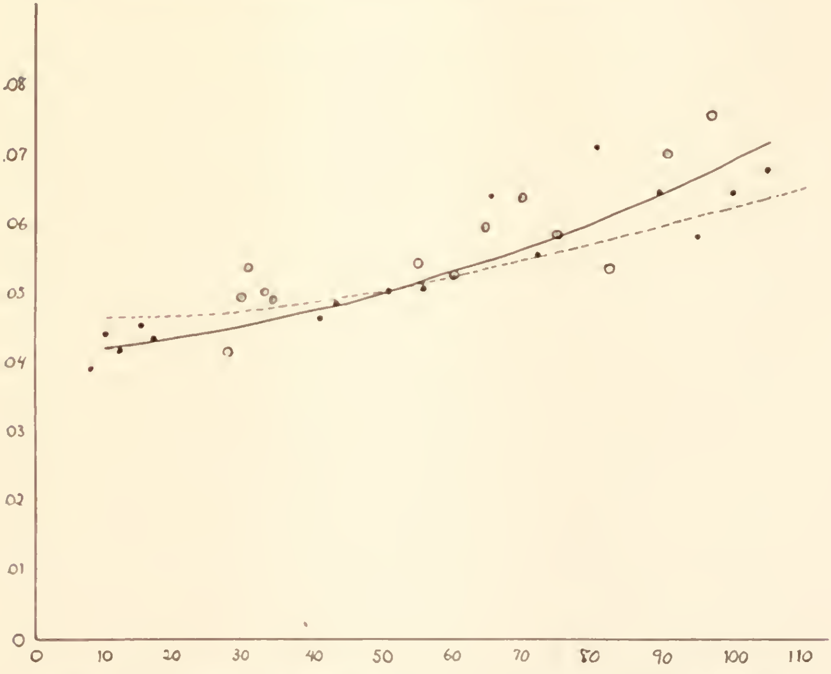

CHART 3. Influence of starvation on carbon dioxide output in *Necturus*. Abscissa, number of days starved; ordinate, CO_2 in milligrams per gram of body weight per hour. Dots refer to *Necturus* No. 4; circles to *Necturus* No. 7. The broken curve is based on the "standard weight" figures in Table VI.

TABLE VI.

EFFECT OF STARVATION ON CARBON DIOXIDE OUTPUT OF *Necturus*.

Necturus 4.				*Necturus 7.*			
Days Starved.	Weight, Gm.	CO_2, Mg.	CO_2 Based on Standard Weight.	Days Starved.	Weight, Gm.	CO_2, Mg.	CO_2 Based on Standard Weight.
8	139	0.0381	0.0420	27	140	0.0417	0.0463
10	139	0.0439	0.0484	29	136	0.0496	0.0535
12	139	0.0422	0.0465	31	136	0.0539	0.0582
15	138	0.0451	0.0498	33	136	0.0502	0.0542
17	138	0.0435	0.0480	34	136	0.0485	0.0522
41	129	0.0466	0.0477	55	127	0.0546	0.0550
46	127	0 0485	0.0489	60	127	0.0527	0.0531
51	127	0.0509	0.0513	64	121	0.0596	0.0572
56	123	0.0511	0.0499	70	121	0.0636	0.0611
66	121	0.0640	0.0615	75	121	0.0586	0.0562
72	120	0.0559	0.0532	82	115	0.0536	0.0489
81	118	0.0713	0.0668	91	113	0.0702	0.0630
89	117	0.0647	0.0601	97	109	0.0756	0.0652
95	117	0.0579	0.0538				
100	113	0.0646	0.0579				
104	112	0.0678	0.0603				

Results on other species are less conclusive regarding the effect of starvation. Extensive determinations were made on *Rana pipiens* with this idea in mind. No changes were noted which could be directly attributed to starvation, except that the extreme inanition previously noted in No. 2 was accompanied by a decided drop in carbon dioxide output at the end. No *Necturi* were carried as far as this. In *Rana palustris* the animals used when near death from starvation showed a much lower carbon dioxide output value than normal starving individuals (about 0.1660 as compared to 0.2730). In *Rana clamitans* the starved individual gave a lower result than the one less starved. With *Amphiuma* No. 2, the carbon dioxide elimination decreased with the weight. It seems, then, that in all species studied except *Necturus* starvation resulted in decreased carbon dioxide output per unit weight, but evidence is offered to show that the opposite is true of this species.

3. Reference to Table VI. will show that there are rather large daily fluctuations in the carbon dioxide output of *Necturus*. Daily records for individuals of other species show the same sort of variation. The possibility is not eliminated that these fluctua-

tions are the result of differençes in the aëration rate. This factor could not be accurately controlled by the apparatus used. However careful observation and comparison of the results of over 250 aërations has convinced the writer that the variations noted are not the result of differences in aëration rate. Many results were discarded in which there was good reason to believe that an abnormally slow aëration had produced a low result. Daily temperature records of the room in which the experiments were carried on were made, and these records show no fluctuations which could possibly account for the daily variations found in the results for individual animals. It is tentatively concluded that we are dealing with unexplained variations in the metabolism of the animal.

4. Sex is responsible for some minor variations in the results. It is known that basal metabolism proceeds at a higher rate in men than in women. The same appears also to be true of Amphibia. If comparable weights be chosen, it is found that *Necturi* female No. 4 is lower than male No. 7, and that female No. 8 is lower than male No. 9. *Rana palustris* females Nos. 2 and 4 are lower than male No. 3.

5. Disease was responsible for some aberrant results in *Rana pipiens*. Several of the animals (Nos. 1, 3, 7 and 8) died of a disease which the writer has called "lymph œdema." It is accompanied by an accumulation of lymph, or water, in the subcutaneous lymph sinuses, leading to a marked increase in weight and a swollen appearance. In the later stages this was invariably accompanied by capillary bursting in the skin and muscles. On autopsy the liver was spotted and the spleen enlarged and crowded with blood. All the animals that died from such œdema showed a sharp rise in carbon dioxide production at the onset of the symptoms. This production remained high until death in spite of the increased weight which would tend to reduce the carbon dioxide per gram weight. For instance No. 3 had a rise from 0.1521 to 0.2427 at the onset of the disease, and died with a production of 0.1798 mg. per gm. of weight in spite of a 25 per cent. increase in weight. No. 6 died from a complication of causes, which included a brain tumor connected with the posterior choroid plexus on the right side, a fatty degeneration which involved the entire right kidney and part of the left,

fatty degeneration of the spleen, lungs and body cavity filled with water, partial paralysis shown by inability to draw up rear legs or to support the body with the right fore leg, and a twisting of the head to the right. This animal showed a fall in carbon dioxide production from 0.1880 down to 0.1239 mg. per gm. per hour, and retained approximately the latter rate until death, in spite of a progressive increase in the severity of the symptoms. Other species did not yield much opportunity to study the effects of disease on metabolism. *Necturus* No. 12 died of fish mould. The determination made on this animal while normal yielded 0.0915 mg. per gm. per hour, while a determination made during the active progress of the infection yielded 0.1354 mg.

6. Motility of the experimental animal may be an important cause of variation in the results obtained. All the animals used were given an hour to get accustomed to the jar before each determination, and there was very little movement in the majority of cases. Salientians would shift their position occasionally, but did very little struggling. A few individuals struggled considerably during the first test made on them. The results of such experiments were discarded. It is interesting that the struggling resulted in approximately doubling the basal rate of carbon dioxide production. *Caudata* struggled very little or not at all.

A few trials were made using curare, which paralyzes the muscles, to see whether more constant results could not be obtained. It was found that the carbon dioxide production of *Rana pipiens* is thus reduced about 25 to 35 per cent., but the daily variations persist. The method described by Lund (1919) of placing the respiring animal in the jar with the barium hydroxide (suspended from the stopper in a basket) was tried on curarized animals. The results checked fairly well with those obtained on the same animals by the aëration method, but it was found that a considerable error is introduced by the necessity of removing the animal from the jar at the end of a measured time; a procedure which stirs up the air in the jar and causes loss of carbon dioxide.

VI. General Comparative Results.

A. Comparison of Classes of Vertebrata.

Table VII. has been prepared to show the cell size variations between the various classes of vertebrates, as indicated by measurements made on corpuscles from a selected representative of each class.

Table VII.

Red Blood Cell Size in Selected Species of Vertebrata.

Class.	Species.	Red Blood Corpuscles.		
		Length.	Width.	Area.
Amphibia......	*Rana pipiens*	20.494	13.153	422
Reptilia ...	*Crotalus adamanteus*	19.0	11.0	349
Pisces........	*Ambloplites ruprestris*	12.37	8.13	158
Aves........	*Gallus domesticus*	12.3	6.7	130
Mammalia ..	*Homo sapiens*	7.9[1]	7.9	98

The frog is below the average of red blood cell size for Amphibia, and the rock bass is below the average for fish. Man is above the average for mammals. The chicken and rattlesnake are near the average for their respective classes. The arrangement of the classes in order of increasing activity would result in the same order as that in Table VII. The avian corpuscle is nucleated, while that of the human is not. There is less difference in bulk of hemoglobin between the two than the measurements would indicate.

B. Comparison of Different Species of Amphibia.

In Table VIII. the species used in this study are listed in order of their red blood cell size, and columns are filled in for weight and carbon dioxide production. All the figures are averages of the detailed results recorded in Table I.

A study of Table VIII. shows that the general trend is clearly in the direction of increasing carbon dioxide output with decreasing cell size, but the results are complicated by the fact that the species with large cells are also large in size. To eliminate the wegiht factor, representatives of several species have been chosen which have comparable weights, and the results from these

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[1] Taken from Gulliver (1875).

TABLE VIII.

AVERAGES OF RED BLOOD CELL MEASUREMENTS AND CARBON DIOXIDE OUTPUT BY AMPHIBIA.

Species.	Weight, Grams.	CO_2 Mg. per Gm. per Hr.	Red Blood Corpuscles.		
			Length.	Width.	Area.
Amphiuma means	1,013.6	0.0390	61.79	36.696	3,561
Necturus maculosus	101.6	0.0814	53.416	28.617	2,401
Cryptobranchus allegheniensis			41.12	23.16	1,496
Diemyctylus viridescens			29.64	17.76	827
Rana catesbiana	511	0.07001	25.66	14.184	572
Rana clamitans	14	0.1576	22.98	11.916	429
Rana pipiens	38	0.1632	20.494	13.153	422
Rana palustris	3.3	0.1820	20.379	12.683	406
Hyla pickeringii			19.44	11.2	342
Acris gryllus			17.76	10.88	304
Chorophilus nigritus			17.96	10.04	283
Bufo americanus	6.4	0.1950	16.004	9.684	244

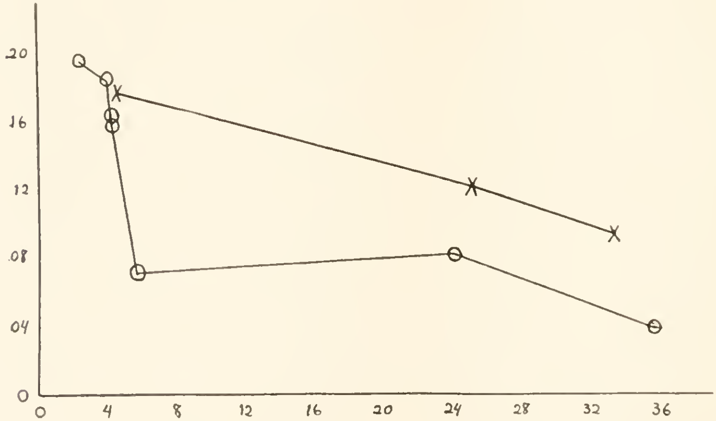

CHART 4. Cell size and carbon dioxide output in Amphibia. Abscissa, area of red blood cells in hundreds of square micra; ordinate, CO_2 in milligrams per gram of weight per hour. Curve based on circles represents the averages taken from Table VIII.; curve bases on *X* represents the individuals recorded in Table IX.

individuals have been recorded separately in Table IX. On Chart 4 are plotted two curves, one based on Table VIII., showing the general upward trend of carbon dioxide output with decreasing cell size, but obviously complicated by the weight factor; the other based on Table IX., showing the even curve which is obtained when weight variations are eliminated.

TABLE IX.

RED BLOOD CELL SIZE AND CARBON DIOXIDE PRODUCTION OF INDIVIDUALS WITH COMPARABLE WEIGHT.

Species and No.	Weight, Grams.	CO_2, Mg.	Red Blood Corpuscles.		
			Length.	Width.	Area.
Amphiuma means, 2	41.4	0.0920	60.9	33.8	3,329
Necturus, 16	44.3	0.1208	50.38	31.66	2,505
Rana pipiens, 3	45.8	0.1768	21.08	14.19	470

The results recorded in Tables VIII. and IX. and in Chart 4 furnish conclusive evidence that there is a correlation between the degree of activity of a species as determined by its carbon dioxide output and the size of its red blood cells. The physiological necessity for such a correlation lies in the necessity for having sufficient surface to allow for the exchanges which take place between surface and interior. If the exchange is rapid, the surface must be large, and this enlargement of surface is brought about by having the mass divided into smaller packets.

VII. SUMMARY AND CONCLUSIONS.

1. Cell size has been measured in a number of species of Amphibia by measuring the dimensions and computing the area of red blood corpuscles. Metabolic activity was measured by collecting in barium hydroxide the carbon dioxide produced by the animal, and computing the carbon dioxide in milligrams per gram of body weight per hour.

2. Comparison of the measurements of red blood corpuscles with measurements published by other authors indicates that the measurements obtained by the present writer are too low. The cause of these low results is unknown.

3. Controls on the method used in determining carbon dioxide output indicate that this method was not extremely accurate, but sufficiently so to allow for making comparisons between the animals used. The chief sources of error were loss of carbon dioxide due to incomplete absorption, and inability to maintain a constant rate of air flow. Indirect evidence that the method is approximately correct is obtained by comparing actual results.

4. There is shown to be a size variation between the corpuscles

of the same individual, and a variation in the average size from different individuals of the same species.

5. A number of factors tend to complicate the results on carbon dioxide production. The most important is body weight, the smaller animals producing more carbon dioxide per unit of weight than the larger animals. This factor becomes especially important in making comparisons of different species, because species vary quite widely in their average weight.

Starvation in *Necturus* seems to cause an increase in the carbon dioxide output per unit of weight, even when all results on an individual are reduced to a constant weight value. In the other species studied starvation seems to lead to a decrease in carbon dioxide production.

Other factors complicating carbon dioxide output results are daily variations in the metabolism of the individual; sex, the male producing slightly more than the female; disease, usually resulting in an increased production, a fact which suggests the fever response in man; and the movement of the animal. This last factor may become of extreme importance if the animal is active, but an attempt has been made to eliminate such results by keeping the animals quiet and discarding results of determinations made on actively moving animals.

6. The conclusion that cell size varies inversely with metabolic activity is justified by the evidence presented. This is shown in a general way by comparing classes of Vertebrata, and more specifically by detailed results on twelve species of Amphibia.

The writer wishes to express his gratitude to Dr. M. F. Guyer, under whose direction the work was undertaken, and to Dr. A. S. Pearse and Dr. H. C. Bradley for their many helpful suggestions and criticisms.

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BIOLOGICAL BULLETIN

RESPIRATORY DIFFERENCES ALONG THE AXIS OF THE SPONGE *GRANTIA*.

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Differences in the rate of respiratory metabolism along the principal axis have now been demonstrated for a number of animals: for the hydroid Corymorpha (Child, '23, Hyman, '23*a*), for the medusa Cassiopaea (McClendon, '17), for Planaria (Hyman, '23*b*), and for several annelids (Hyman and Galigher, '21). Recently Shearer ('24) has reported similar results for the chick embryo and the earthworm. Unfortunately in Shearer's work regions of very different morphological constitution were compared and it is therefore doubtful if his results can be regarded as lending support to the physiological gradient conception. For example anterior and posterior halves of the chick embryo in the stages studied by Shearer differ enormously in their content of nervous tissue, due to the presence of the brain and chief sense organs in the anterior half. The much greater respiratory rate of the anterior half reported by Shearer is probably largely due to the greater proportion of nervous tissue which it contains. Similarly in the earthworm the mature head is morphologically and functionally different from the rest of the body and respiratory differences between it and other regions must be in part due to such specific differences. In brief, Shearer's measurements concern not the gradient itself but the secondary differentiations associated with the gradient. Shearer also seems to be unaware of the existence in annelids (and in the early embryos of vertebrates) of the double type of gradient (Hyman and Galigher, '17) and the pieces of the earthworm which he compared were consequently not correctly chosen and serve neither to prove nor disprove the existence in this animal of the type of gradient which we have described for it. The acetone powder experiments do not, in my opinion, answer these objections. If the substance in such powders which absorbs oxygen is essential to respiration then it is necessarily true that types of tissues which have a high respiratory rate must contain a higher percentage of the substance. It has been repeatedly emphasized that direct proof of axial respiratory differences can be obtained only through the comparison of pieces of like morphological constitution and through the elimination of various functional factors which affect metabolic rate. It is to be hoped that Shearer will repeat these experiments with more suitable material. Such material is necessarily limited to the lower invertebrates or to the very early embryonic stages of higher forms.

The sponge *Grantia* seemed to me to constitute very favorable material for a further test of the reality of axial metabolic differences. It has the same morphological constitution throughout except at osculum and base and is more or less definitely polarized. The work was performed at the Marine Biological Laboratory at Woods Hole during the summer of 1924.

1. Method of Determining the Oxygen Consumption.—For two or three years I have been trying to devise an apparatus suitable for the study of the oxygen consumption of small organisms by Winkler's method. The method finally adopted owes its origin to a device described by Osterhaut and Haas ('17). They first suggested an apparatus separable into two pieces, one part to contain the organisms and the other part for analysis. Their apparatus is, however, clumsy to manipulate and for large animals or large amounts of material the method used by me for many years of siphoning off the sample is very much simpler and entirely satisfactory, as proved by checks. The device proposed by Osterhaut and Haas to prevent exposure to air in adding the reagents is in my opinion wholly unnecessary unless one is dealing with water of very low oxygen content. In working with small organisms, a very much smaller apparatus is required. This naturally reduces the size of the sample of water available for analysis. This difficulty is overcome by using a smaller quantity of the reagents and a more dilute thiosulphate solution for the final titration, as also suggested by Lund ('22).

The apparatus which I have been using has the following simple construction, illustrated in the accompanying figure. It consists of a Pyrex test tube *A*, without rim, 100 mm. long by 10–12 mm. diameter, capacity about 10 cc. This is surmounted by a piece of rubber tubing *B*, into which fits a short length (about 40 mm.) of Pyrex tubing *C* of the same diameter as the tube *A*. Over the end of *C* is another piece of rubber tubing *D*. The rubber tubing must fit tightly over the glass and if necessary *B* can be wired to *A* and *D* to *C*, but not *C* to *B*. A number of such outfits should be prepared. For the water blanks only the parts *A* and *B* are necessary.

The apparatus is used as follows. In those tubes which are to contain the animals all four pieces must be fitted together as in the figure and *C* must be shoved close to but not in contact with *A*. All of the tubes to be used in one experiment, including the tubes for the blanks, are filled by siphon with the same water from an elevated receptacle. The siphon should reach to the bottom of the tube and the water be allowed to flow out of the top for some time. The animals to be used can be placed in the tubes before they are filled with water if of suitable nature, or if not, can be put in after the filling. All tubes are then closed by screw clamps around *D* in the experimental tubes, or *B* in the blanks. The tubes are then placed in a suitable water bath kept at constant temperature. Those containing the animals should be agitated at intervals, in the case of non-motile animals, to prevent an accumulation of metabolic products and exhaustion of the oxygen around them. When it is desired to conclude the experiment, each experimental tube is inverted several times to insure uniform distribution of its oxygen content and is then finally inverted, the animals being brought by gravity into the section *C* plus *D*. A screw clamp of suitable size is then rapidly placed around the tubing *B* over the small interval left between *A* and *C*. On screwing the clamp, *A* and *C* will be found to move apart readily and the walls of the tubing *B* are at the same time drawn together by the negative pressure thus

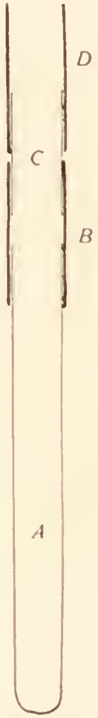

FIG. 1.

developed. One should never try to pull the sections *A* and *C* forcibly apart. After screwing the clamp tightly, the sections *C* plus *D* are withdrawn from *B*, the apparatus being held inverted during the whole of the procedure to prevent the spilling of the contents of *C* plus *D*. The contents are immediately poured into a small graduated tube and the volume noted. Graduated centrifuge tubes have been found very convenient for this purpose. A little practice with the apparatus is required to prevent the spilling of the water from *C* plus *D* and a tube should be at hand to receive this water just as *C* is withdrawn from *B*. The portion *A* plus *B* is now analyzed by Winkler's method and the blanks which consist of only *A* plus *B* are analyzed at the same time. The clamp around *B* in each tube is open and 0.1 cc. of each of the reagents used in Winkler's method is added. The clamp is then closed, the contents shaken, the precipitate allowed to settle as usual, and after again opening the clamp, 0.1 to 0.2 cc. concentrated HCl added carefully so as not to disturb the precipitate. The clamp being again closed, the tube is shaken to dissolve the precipitate and the contents are then poured into a small evaporating dish and titrated with sodium thiosulphate. The latter should be about 1/500 normal. With greater dilution of the thiosulphate the end point becomes uncertain. The volume of the tube *A* plus *B* must then be determined. If care is taken not to move the clamp around *B* during the preceding operations, the tube *A* plus *B* can be filled with water of the same temperature as that obtaining during the experiment, the clamp closed, excess water at the top removed, and the contents then poured into a graduated tube. In my experience the volume of *A* plus *B* and *C* plus *D* must be determined at each experiment as they vary slightly owing to variations in the position of the clamp when the two portions are separated. One must remember to subtract 0.2 cc. from the volume of *A* plus *B*, to compensate for the loss of this amount of water when the reagents are added.

In calculating the results, it must be borne in mind that only the portions *A* plus *B* are analyzed while the animal respired from *A* plus *B* plus *C* plus *D*. The thiosulphate equivalent of each cc. of *A* plus *B* is calculated and from this the thiosulphate equivalent of the entire apparatus is obtained. It is thus necessary to know the exact volume of *A* plus *B* and *C* plus *D*. The oxygen content at the beginning is determined in the same way by calculation from the water blanks and is brought by ratio to the same volume as the experimental tube. A simple subtraction then gives the oxygen consumed by the animals in terms of thiosulphate. To find the oxygen equivalent of the thiosulphate the latter must of course be standardized. A number of methods are given in any text on quantitative analysis. If the oxygen consumption in terms of volume is desired, as is usually the case, then it is desirable to conduct all of the experiments at the same temperature and to determine the volume of the tubes for this temperature. The oxygen equivalent of the thiosulphate for any given temperature can be determined from data on the volume of oxygen at different temperatures given in handbooks of physical constants.

The apparatus can be modified in various ways to suit the type of animal employed. For Protozoa the tube is closed, after filling with water and adding the animals, not by means of a clamp but by means of a glass plug. This is inserted deeply into *D* and must be firmly wired in. At the end of the experiment the tube is placed in the centrifuge in the inverted position, the bottom of *A* pointing towards the axis of the centrifuge. By centrifuging, the Protozoa are driven into the section *C* plus *D*, which is then removed as already described. For larger animals the size of the apparatus can be increased or in place of the test tube *A* a small flask of desired content can be substituted. In such cases, the amount of the reagents used and the dilution of the thiosulphate should be adjusted to the size of the sample obtained for analysis. The apparatus is not suitable for animals which cling firmly, such as planarians.

The method is naturally not as accurate as the regular Winkler method. By the analysis of duplicate samples I have found that the oxygen content of 15 cc. of water—about 0.07 to 0.08 cc. at air saturation and room temperature—can be determined with an error of about 0.002 to 0.003 cc. This makes an error of some 3 to 4 per cent. while in the regular Winkler procedure, with samples of at least 100 cc., the error is less than 0.5 per cent. The method is thus chiefly of value for comparative work.

2. Method of Determination of Carbon Dioxide Production.—For this purpose the simple method first suggested by Haas ('16) was employed. A set of standard tubes containing phenol red solutions covering the range of hydrogen ion concentration from pH 6.8 to 8.4 must be at hand. These are now sold by dealers in chemical supplies. For the experiments tubes of the same dimensions are necessary. The animals to be tested are placed in such tubes and those to be compared must be of the same weight. Phenol red in powder form is added to sea-water until the density of color is the same as that in the tubes of the standard set. An equal volume of this water is then added to each of the tubes containing the animals and the tubes then sealed with paraffin. If the inside of the tube above the water is wiped dry, paraffin will adhere to it firmly and the melted paraffin can be then added directly onto the surface of the water. The sealed tubes are placed in a water bath at constant temperature and the changes in tint due to the production of carbon dioxide by the organisms are recorded in terms of pH by comparison with the standard tubes. There is of course some error (probably about .2 pH) in working with sea-water unless the standard sets have been especially prepared for such work. This error is of no consequence in comparative work.

3. Material and General Procedure.—Only freshly collected sponges were employed and these were used as soon as brought in by the collectors. Only the cleanest and most perfect specimens were used; those selected were placed in a dish of clean sea-water and repeatedly squirted about with a pipette to free them as far as possible from foreign materials clinging to their surfaces. Unfortunately in the case of sponges it is not possible to determine by inspection whether the specimens are in good physiological condition or not. The selected specimens were placed on a glass plate, osculum and base cut off and discarded, and the body then cut into two nearly equal halves. These pieces were then placed in the tubes for the respiration tests.

It was of course necessary to weigh the pieces. They were gently rolled about on hard filter paper until they no longer wet the paper, then transferred to small weighing bottles, previously weighed, and weighed to the fourth place. In the case of the oxygen consumption tests, the pieces were weighed after the conclusion of the experiments. For the carbon dioxide production tests, it is necessary to weigh the pieces in advance since by this method only pieces of equal weight can be compared. The sponges were cut into slightly unequal portions, and the smaller portion weighed first. The larger portion was then weighed and small pieces removed from it until its weight equalled that of the other piece. After such handling it was thought advisable to allow the pieces to stand in water for two or three hours before beginning the tests and this was always done.

The sea-water used in all of the experiments was kindly furnished to me by Dr. A. J. Goldfarb. This water was collected from the end of the Bureau of Fisheries pier at Woods Hole and stood for several days prior to its utilization to allow debris to settle. This water contained no organisms visible to the eye but no doubt some bacteria were present. As the blanks however are allowed to stand as long as the experimental tubes before analysis, this possible source of oxygen loss is cancelled out. The water was thoroughly aërated before use and was thus saturated with air at the beginning of every experiment.

In nearly all experiments two pieces of sponge were placed in each tube, such pieces being of course from the same level of the sponges concerned. Thus for each experiment two sponges were selected and cut and the two apical halves placed in one tube, the two basal halves in the other. The tubes were then filled by siphon as already described. In some cases they were filled first and the pieces of sponges dropped in afterwards. In the experiments on carbon dioxide production, the pieces were placed in the tubes and with a pipette a definite amount of sea-water colored with phenol red run into each tube. At first five cc. of water were added to each tube but the carbon dioxide production was found to be so slow that later only two cc. were employed.

The control of temperature was difficult at Woods Hole. Owing to the lack of constant temperature apparatus, the experiments had to be run at room temperature. At the beginning of each oxygen consumption experiment, the water and water bath were allowed to come to room temperature and thereafter the bath was kept at this temperature by adding warm or cold water as the case might be. The carbon dioxide experiments, however, ran over such long periods of time in many cases, that the temperature could not be kept constant by personal attention and varied during the night with changes in the air temperature.

4. *Results.*—The results of the oxygen consumption experiments are given in Table I. Thirteen experiments were performed, of which one (No. 10) was lost. The table shows the oxygen content of the whole tube ($A + B + C + D$) at the

TABLE I.

OXYGEN CONSUMPTION OF APICAL AND BASAL HALVES OF THE BODY OF THE SPONGE *Grantia.*

Duration of each experiment three hours. Temperature in different experiments 21 to 23° C. Oxygen given in cubic centimeters.

No. of Exp.	Kind of Piece.	O_2 Content at Start.	O_2 Content at End.	Oxygen Consumed.	Weight.	O_2 Consumed per Gram per Hr.
1	Apical	0.080	0.072	0.008	0.0193	0.138
	Basal	0.082	0.071	0.011	0.0265	0.138
2	Apical	0.077	0.061	0.016	0.0345	0.154
	Basal	0.074	0.057	0.017	0.0360	0.157
3	Apical	0.077	0.063	0.014	0.0285	0.163
	Basal	0.069	0.059	0.010	0.0319	0.104
4	Apical	0.074	0.053	0.021	0.0578	0.121
	Basal	0.074	0.049	0.025	0.0730	0.114
5	Apical	0.082	0.070	0.012	0.0682	0.058
	Basal	0.077	0.067	0.010	0.0732	0.043
6	Apical	0.080	0.069	0.011	0.0452	0.081
	Basal	0.074	0.062	0.012	0.0536	0.074
7	Apical	0.077	0.069	0.008	0.0310	0.086
	Basal	0.077	0.069	0.008	0.0482	0.056
8	Apical	0.074	0.052	0.022	0.0442	0.166
	Basal	0.075	0.055	0.020	0.0452	0.147
9	Apical	0.074	0.055	0.019	0.0388	0.163
	Basal	0.071	0.060	0.011	0.0428	0.086
11	Apical	0.074	0.053	0.021	0.0510	0.137
	Basal	0.075	0.055	0.020	0.0581	0.114
12	Apical	0.073	0.057	0.016	0.0320	0.166
	Basal	0.066	0.053	0.013	0.0406	0.106
13	Apical	0.072	0.054	0.018	0.0392	0.153
	Basal	0.073	0.051	0.022	0.0558	0.131

beginning and at the end of each experiment. The former is determined by calculation from the control blanks, and the latter by calculation from the analysis of the portions *A* plus *B* of the experimental tubes, the contents of *A* plus *B* and *C* plus *D* being known. When calculated in this form, the difference between the two values gives the oxygen consumed by the pieces. This divided by the weight and then by three (as each experiment ran for three hours) gives the oxygen consumed per gram of weight per hour. The figure thus obtained seems a little low as compared with the respiration rate of other animals

TABLE II.

RELATIVE CARBON DIOXIDE PRODUCTION OF APICAL AND BASAL PIECES OF THE BODY OF THE SPONGE *Grantia*, IN TERMS OF TIME REQUIRED TO CHANGE PHENOL RED FROM pH 8.0 TO 7.5.

h., hours; m., minutes.

No. of Exp.	Kind of Piece.	Weight, Grams.	Time for Change from 8.0 to 7.5.	Remarks.
1	Apical Basal	0.051 0.058	5 h., 50 m. 9 h., 40 m.	5 cc. sea-water used.
2	Apical Basal	0.0212 0.0234	21 h., 30 m. 25 h., plus	5 cc. End point of basal piece not exactly determined.
3	Apical Basal	0.0314 0.0318	20 h., 40 m. 24 h., 55 m.	5 cc.
4	Apical Basal	0.0217 0.0214	21 h. 16 h.	4 cc. Repeated with same result.
5	Apical Basal	0.019 0.019	26 h., 15 m. 26 h., 15 m.	5 cc. Almost no difference between them at any time.
6	Apical Basal	0.0182 0.0184	15 h., 40 m. 17 h., 30 m.	4 cc.
7	Apical Basal	0.0179 0.0174	5 h., 15 m. 8 h.	2 cc.
8	Apical Basal	0.0212 0.0226	6 h., 25 m. 8 h.	2 cc.
9	Apical Basal	0.0313 0.0300	5 h., 30 m. 9 h.	2 cc. Basal piece in advance on next day.
10	Apical Basal	0.0218 0.0234	8 h., 10 m. 9 h., 40 m.	2 cc. Even next day.
11	Apical Basal	0.0112 0.0124	17 h. 19 h.	2 cc. No difference between them until after 4 hrs.

—it is about half of that of Planaria—but it must be remembered that a considerable portion of the weight of the sponge is due to the lifeless spicules.

Of the twelve experiments presented in Table I., the rate of oxygen consumption is markedly greater in the apical piece in five cases (Nos. 3, 5, 7, 9, and 12), slightly greater in five cases (Nos. 4, 6, 8, 11, and 13) and about equal to that of the basal piece in two cases (Nos. 1 and 2). In experiments 4 and 6 the difference between the apical and basal pieces is so slight as probably to be of no significance.

The experiments on carbon dioxide production yield about the same result. In nine cases, the apical pieces produce carbon dioxide at a faster rate than do the basal pieces, in one case (No. 5) the rate is equal and in one case (No. 4) the result is the reverse. The advantage in favor of the apical piece is not very great in Nos. 10 and 11.

These findings are in agreement with the electrical differences previously discovered in this sponge (Hyman and Bellamy, '22). It was found that in the majority of individuals the oscular end is electropositive (internally) to more proximal regions, but that in some individuals this potential difference is absent or may even be reversed. That these electrical differences are correlated with the metabolic differences is a view which I have held for a number of years. It is presumable that those individuals in which electrical or metabolic gradients are lacking are in poor physiological condition.

Finally attention may be called to the relation between rate of oxygen consumption and size (weight). In general, the greater the weight of the pieces, the lower is the rate of oxygen consumption. This inverse relation between size and respiratory rate seems to be general throughout the animal kingdom (cf. Hyman, '19).

5. *Summary.*—In the majority of cases, apical pieces of the body of the sponge *Grantia* consume oxygen and produce carbon dioxide at a higher rate per unit weight than do basal pieces. This result furnishes further evidence in favor of the axial gradient conception.

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THE DISTRIBUTION OF CERTAIN INSECTS OF REVERSED BEHAVIOR.*

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The recent researches of Loeb, Holmes and other students of animal behavior as well as the work of various entomologists on the reactions of insects to definite environmental factors, has given us a mechanistic interpretation of insect activities very different from the anthropomorphic interpretation of the earlier students. Shelford, Dean and others have related many insect adjustments in behavior with remarkable exactness to specific conditions of light, temperature, humidity, etc.

These reactions are frequently quite specific but many of them are the same for all the species of a genus. As, for instance, all species in a genus are nocturnal or all are aquatic. Such a generic tropism usually defines the generic habitat in a broad way. Within this general habitat the individual species will have individual habitats limited by other tropisms. Apparently the generic tropism that defines the generic habitat is seldom modified for any individual species enough that such a species may exist outside the generic habitat. But apparently a complete reversal of a generic tropism is more likely of occurrence than any lesser modification. When such occurs in a genus the individual species possessing this reversed generic tropism has entrance into an environment closed to all other members of its genus.

The writer has come across two species of insects, one a dragonfly, the other a mayfly, in which a reversal of one or more of the tropisms normal to the other species of the same genus has permitted the entrance of these reversed species into environments not open to the normal members of the genus. These finds have opened up so many interesting problems in behavior and distribution that they are well worth presenting in some detail.

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*Contribution from the Department of Zoölogy and Entomology, Ohio State University, number 79.

The first of these that was found is *Æshna nevadensis* Walker of the Sierra upland of central California.[1] This is a large, pond and shallow lake dragonfly that lives at elevations of 5,000 to 9,000 feet. It is known only from the southern Sierra Mountains. It has been recorded from Walker Lake, Mono Co., California, at 7,700 feet elevation, Hardin Lake, Tuolumne Co., at 7,775 feet elevation and from Elizabeth Lake, Yosemite National Park at the great altitude of 9,000 feet. The writer found it at its optimum development in shallow, weed-filled lakes on the divide between Lake Tahoe and the Rubicon River (Calif.) where at an elevation of 7,000 feet it dominated the subalpine dragonfly life. Here in an open, meadow-like mountain pass, fairly level for about two miles, lie four shallow lakes, two flowing into Lake Tahoe and the desert drainage, while the other two empty into the Rubicon River and the Pacific drainage. On both sides rise granite crags for a thousand feet above the lakes, their lower parts green with firs, while their gray upper slopes are blotched white with fields of snow. Three of the lakes are covered with yellow flowered pond lilies and are fringed with green sedges among clumps of silver gray willows. These shallow lakes are warm because of the black peat bottom and long hours of sunshine. They support great numbers of a few species of insects—such as are able to withstand the long winters and cold nights at this elevation, as this is a subalpine situation with nightly temperatures at or near freezing. Eight other species of dragonflies are common here. These are northern forms that occur in the mountains of Oregon at 3,000–4,000 feet elevation and in British Columbia at sea level.

The habitat of *nevadensis* appears to be entirely above that of all other species of North American Æshnas. It appears as strayed individuals at 4,500 feet but does not appear in numbers until an elevation of 6,000–7,000 feet is reached. From here up to 9,000 feet it appears at its optimum development. No other Æshnas have been found regularly at these higher altitudes. From about 4,500 feet down to sea level, however, Æshnas are a constant element of the North American Odonate fauna. Twenty or more lowland species have been described from north of Mexico while there are actually seven or eight species living about the lower slopes of the Sierras. Thus *nevadensis* has a habitat entirely above or outside of that of the other North American members of the genus.

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[1] Kennedy, *Proc. U. S. Nat. Mus.*, Vol. 52, pp. 483–635.

We do not know the behavior complex of *Æshna nevadensis* in enough detail to speculate on all the adjustments in reactions necessary to adapt this species of a lowland genus to a highland habitat. It shares with all other Æshninæ, and without any change, a positive reaction to shining water surfaces when the sexual instinct is not overbalanced by hunger. This is a reaction through the eye as *Anax*, a related genus, was found reacting just as positively to the glistening surfaces of the crude oil pools of the Bakersfield (Calif.) oil field where hundreds of *Anax junius* perished while mating and trying to oviposit in the crude oil. When hunger predominates over the sexual impulse the reactions change so that the Æshnas fly away from the water on hunting trips into the surrounding territory. They fly away from the water and roost on trees when the minimum flying temperature is reached, also at twilight on warm evenings when the minimum flying light is reached. The minimum flying light varies greatly with the different species as some will still fly when it is so dark to the human eye that the dragonfly can be seen only when it is outlined against the sky or some white surface.

As ponds for oviposition are the same on the Sierra upland as at sea level, we find the reactions of *nevadensis* while under the sexual impulse practically identical with the similar reactions of the lowland species. The adjustments to the upland come in the reactions of the insects when hunger and other impulses outweigh the sexual impulses, and have nothing to do with the coursing of the males and females over the surface of the water while mating and ovipositing.

Two of these adjustments to the conditions found in these high altitudes are quite obvious. First the hunting individuals react negatively to the warm stratum of air next the ground so that, except early in the morning when the ground stratum of air is still cool, they hunt high off of the ground flying from fifteen to one hundred and fifty feet in the air. It is a tree-top species. This positive reaction to cool air probably explains the attraction of *nevadensis* to this alpine habitat. All other species of Æshna, when hunting, react negatively to the cooler situations of the habitat, the warmer the place the better, until 100–105° is reached when some species begin to become inactive.

The second obvious adjustment necessary is in the time of emergence. All lowland species of Æshna, as far as known, emerge at night, those the writer has reared, *umbrosa* and *constricta*, emerging at or near midnight. This appears to be an adjustment of value in that the imagoes have hardened sufficiently by daylight for flight and so escape the blackbirds and other marsh fowl that enjoy soft freshly emerged insects. But at an elevation of 7,000–9,000 feet with snow fields spread about, the nightly temperatures are never far above freezing and frequently below, making night emergence precarious as the young dragonfly would be too chilled to crawl out of its nymphal skin. Here a second adjustment to this elevated habitat appears. The emerging nymph of *nevadensis* instead of being negatively geotropic in the dark is negatively geotropic in the light, so that the winged adult emerges in the broad daylight when the temperature is high enough to insure a successful withdrawal from the nymphal skin. The writer found numerous individuals emerging in the bright sunshine in the early afternoon hours.

Thus we see that *Æshna nevadensis* has left the general warm lowland environment of the genus and has entered an entirely different region through having two of the tropisms normal to Æshna reversed.

While in the mountains of eastern Tennessee last spring, the writer discovered a mayfly, *Ephemera guttulata* Pictet, which appears to occupy a habitat entirely different from that of any other species of Ephemera. It too appears to have certain of its reactions the reverse of those of the other species of this genus.

Ephemera guttulata is a most interesting mayfly in several ways. It is one of our large mayflies. Its wings are so heavily clouded that at a little distance they appear almost black, especially as contrasted against the abdomen, which is immaculate snow-white. This bizarre insect lives in the smaller of the perennial, spring-fed mountain torrents that flow down the higher of the Eastern Tennessee Mountains. On Chilhowee Mountain these streams pour down deep V-shaped ravines over beds of small stones and coarse grit, in a succession of miniature waterfalls, for they descend at a rate of several hundred feet to the mile. These mountains are covered with pines on their high, dry ridges but the deep ravines between these ribs of pine woods are filled with a dense growth of deciduous timber so that the torrents are heavily shaded by tall trees in their whole course.

The burrowing larva of *guttulata* lives in the meager areas of coarse sand and muck found in the little basins below the waterfalls. The subimago emerges during the day. Those the writer observed came out on dull cloudy days. These fly out of the shade over the stream, through the surrounding brush and up to the better lighted areas of the hill side where they rest in the full light. The dull gray subimago then sheds a thin skin and comes out a fully developed imago with its brilliant black and white colors and its sexual maturity. No observations were made as to whether this occurred on the day of emergence or the following day. Because of the few subimagoes seen it probably occurred the same day as the emergence. Unfortunately also, no mating dances were seen. These probably occurred among the tree tops, in the deep dusk, just before egg laying began.

When the evening twilight had deepened to the point where it became difficult to pick one's way along the streams, the females of *guttulata* would appear over the little pools hurrying back and forth about a foot above the surface of the water apparently laying eggs. Their conduct was more like that of female dragonflies than like the usual hurried visit of the mayfly female dropping all of her eggs in a single effort. No males were caught at these times over the streams.

It was in these flights in the dense twilight gloom of the bottoms of these mountain gorges that the probable value of the bizarre coloration came to mind. The enormous development of the eyes, the evidence of the rudimentary antennæ together with certain experimental work indicate that the major reactions of the mayflies are through the sense of sight. Except for the white abdomen, the mayflies, at the time of these twilight flights, were practically invisible to the observer. These white abdomens, as the *guttulata* females doged about in the gathering darkness, reminded one of the streaks of light of a flight of fireflies. Apparently then this white abdomen is useful to *guttulata* in the mating flights in the deep shade of the mountain gorges as its visibility is very obviously increased by this color pattern. A snuff-colored, lowland Ephemera would be practically invisible under the same conditions. It is interesting to note that of several species of mayflies flying on these streams at this time *guttulata* is the latest on the wing in the evening and flies after the others have ceased. Some of the smaller species fly a full hour earlier, so that, though, of dull colors they are quite visible.

We can check this series of reactions of *guttulata* by a comparison with the reactions of the other species of Ephemera, all of which inhabit either large open streams or lakes. Probably the reactions of *Ephemera simulans*, the common Lake Erie species, are best known. The nymph of this species burrows in the mud of the lake bottom, being obviously negatively phototropic. At the time of emergence it becomes positively phototropic and rises to the light of the sky. At Put-in-Bay this emergence takes place between eight and ten P.M. It sheds its skin as it rises through the ten to thirty feet of water so that on arrival at the surface it bursts out fully winged, when it becomes less positively phototropic and flies toward the dark land. It rests on the shore vegetation until the following evening when it sheds its final skin, becomes sexually mature and at twilight flutters up and down in the mating dance. At this stage it is evidently becoming positively phototropic again. In this twilight nuptial dance it leaves the dark foliage for the more open lighter spaces. The males grasp the females and release them after a contact of a few seconds. The female becomes at once completely, positively phototropic and flies out toward the light surface of the lake to deposit her eggs.

If we compare this series of reactions with those of *guttulata* of the shaded mountain streams, we find that two of the series of reactions of the latter are reversed. *Guttulata* is negatively phototropic as a nymph, is positive as it emerges, but *remains positively phototropic after emergence* as it flies from the heavily shaded creek to the lighter areas above the shade. Further, *after copulation it becomes negatively phototropic* and flies down to the densely shaded torrent to oviposit. Any of the open stream species of Ephemera would react themselves away from the shaded stream when they started to oviposit. So by these reversed reactions *guttulata* is able to occupy a habitat that the normal members of the genus *Ephemera* are not able to occupy, one that is ecologically outside of the general habitat of the genus.

Certain experimental studies in the behavior of mayfly nymphs point to possible explanations of some of this series of reactions. Wodsedalek[2] has demonstrated that the nymph of Heptagenia is negatively phototropic. Also that CO_2 in the water will make a nymph which has been negatively phototropic, reverse its reaction and become positively phototropic. It is then possible that the change in reaction at the time of emergence, when the nymph ceases to burrow and swims up to the light, is due to an accumulation of CO_2 in the nymph after its tracheal system has detached itself from the gills in the last nymphal ecdysis. The shedding of the skin actually starts by a general loosening of the chitin several hours before the final emergence occurs. During this time, if the gills are early detached, much CO_2 could accumulate in the tissues, enough eventually to reverse the phototropism and so cause the nymph to rise to the surface.

On emergence the nymph fills its tracheæ with air and simultaneously becomes negatively phototropic, so that it flies toward dark land. It appears to retain this reaction until the next evening at dusk or for about twenty hours when it mates and the female at once becomes positively phototropic and flies towards the light surface of the open lake. During the intense nuptial dance her tissues have been accumulating CO_2 and in some way the sexual orgasm overbalances the condition, giving the acid condition full sway.

The experiments of Allee and Stein[3] show that the reactions are not as simply explained as they have been sketched in the preceding paragraphs. The actual intensity of the light probably also figures in some of the reactions. Krecker's work[4] in the subimagoes of *Hexagenia*, a close relative, shows that these, in spite of flying to the dark land, are positive to certain bright lamps. Anyone who has seen the snow storms of mayflies that come to the street lamps of the lake ports realizes that the subimago can have a reverse tropism under such conditions.

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[2] Wodsedalek, "Phototactic Reactions and their Reversal in the Mayfly Nymphs of *Heptagenia interpunctata* (Say)," Biol. Bull., Vol. 21, pp. 265–271, 1911.

[3] Allee and Stein, "Light Reactions and Metabolism in Mayfly Nymphs," *Jour. Exp. Zoöl.*, Vol. 26, pp. 423–458, 1918.

[4] Krecker, "Phenomena of Orientation Exhibited by Ephemeridæ," Biol. Bull., Vol. 29, pp. 381–388, 1915.

We are beginning to recognize physiological species among insects—those based on habits and habitats. In parasitic insects particularly we recognize generic reactions to common hosts so that we unofficially recognize physiological genera. There is no reason why we should not, except the expediency of morphological characters. Viewed in this light *Æshna nevadensis* and *Ephemera guttulata* are physiologically outside of their respective genera.

When the writer first thought through the habits and distribution of the North American species of the genus *Æshna*, his conclusion was that the positive thermotropism of the lowland species was perhaps different in degree and distinct for each species, thus explaining the restriction of each species to its specific thermal belt. Thus it appeared, at first, that this difference in positive thermotropism accounted for the fact that some Æshnas lived in hot Arizona, others in the cooler northern states, while still others are restricted to the northern parts of Canada and Alaska. However on investigating further this does not appear to be true.

The restriction of each species to its specific thermal belt is probably not due to a limitation of the positive thermotropism of the adult to the narrow limits of the particular thermal belt inhabited by that species. This is quite contrary to Merriam's theory of distribution by thermal zones.[5]

This distribution of the various species in different thermal zones however is a very striking thing and some of the thermal conditions are easily sketched. Except for *mutata*, *californica* and *multicolor*, which are early spring species, the majority of the species of Æshna are on the wing in August, so the writer has worked out the flying temperatures for August at four points, Yuma, Arizona, St. Louis, Missouri, St. Paul, Minnesota, and Sitka, Alaska. Each of these is representative of the flying conditions for a restricted group of Æshnas. The Yuma temperatures apply to *jalapensis*, *multicolor* and *arida*. The St. Louis temperatures apply to *constricta*, which is the only species broadly distributed across the central states, while the St. Paul temperatures apply to a series of several species such as *interna*, *canadensis*, *umbrosa*, etc. Under the Sitka temperatures we find *sitkensis*, *subarctica* and *septentrionalis*. Such species as *palmata* and *constricta* are found in two or three of these zones.

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[5] Merriam, "Life and Crop Zones of the United States," Bull. U. S. Biol. Survey, No. 10, pp. 1–79, 1898.

To define the flying temperatures the mean maximum and mean minimum day temperatures for August have been taken from the Weather Bureau Report for the above stations.[6] Ten degrees was then added to the mean maximum day temperature to give the approximate maximum sun temperature, which is the temperature to which the local dragonflies react positively, while the mean minimum day temperature remained unchanged, as it is a shade temperature to which the local dragonflies react negatively. Thus these temperatures show roughly the range of day temperatures which the species of *Æshna* meet during their flight season in each of the general zones represented by the temperature records. These records for August are as follows:

	Mean Maximum in the Sun.	Mean Minimum in the Shade.
Yuma, Arizona	114°	77°
St. Louis, Missouri	96°	69°
St. Paul, Minnesota	90°	60°
Sitka, Alaska	72°	45°

From the above it is obvious that the temperatures during flight do differ greatly for the various species of *Æshna*. However our observations indicate that all lowland Æshnas are always positive to heat so that the higher the temperature, the greater the activity of the insect. Hine's observations[7] on Kadiak Island were that *palmata* was most active on the warmest days. Walker's observations[8] on the Canadian species are that increased temperatures always increased the speed of Æshnas but his observations do not include temperatures above 90°. Somewhere above 90° there may be a limit for this increase of speed as the temperature rises, which limit may be actually reached by some of the southern species on very warm days. The writer [9] has made one interesting observation in this regard. *California* and *multicolor* on very hot days when the temperature is from 100° to 105° take frequent rests, by hanging up in the shade every few minutes. This was noted in the Yakima desert at Sunnyside, Washington. Apparently, for these at least, somewhere in the higher nineties a temperature is reached above which increase is depressing and no longer stimulating. However we can safely say that for all lowland species of *Æshna* an increase of temperature up to 95° increases activity so that all are equally positively thermotropic.

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[6] "Climatology of the United States," Bull. 2, 1906. Temperatures for Yuma, St. Louis and St. Paul. *Monthly Weather Review*, Dec., 1898, p. 549. Temperature for Sitka.

[7] In conversation with the writer.

[8] Walker, p. 33. "The North American Dragonflies of the Genus *Æshna*," Univ. of Toronto, Biol. Studies, Biol. Series, No. 11, 1912.

The thermal distribution of the four groups of species outlined above must then be conditioned through some indirect check on the life history. Some temperature condition of the water for the nymph or the developing egg may be the limiting factor rather than the flying temperature for the imago. Undoubtedly individuals of each species continually fly beyond the limits of the optimum habitat but the offspring of such do not survive or we would have a spreading species. This constant pressure of the species of dragonflies into surrounding but unsuitable habitats was worked out by the writer [10] on Put-in-Bay Odonata in 1922. Until we know the life histories of the various species in minute detail we will not be able to define all of these limiting factors except as we stumble onto them accidentally.

A further conclusion appears indicated from this study. As all Æshnas, except *nevadensis*, have one type of reaction to temperature in the imago, and all the Ephemeras, except *guttulata*, have one type of reaction to light and the reactions of these two odd species are just the reverse of the other species in their respective genera, we may conclude that a given type of insect nervous system can be completely reversed from its usual reaction more easily than it can be modified in a lesser degree. This would appear logical from the theoretical grounds of the mechanics of the nervous system. Because of its minute size, the insect nervous system is characterized by the relatively small number of units (either cells or combinations of cells) that comprise it. Such simple nervous systems obviously cannot produce the extended series of finely graduated reactions that are possible to a more complicated type of system. On the other hand the mechanism is already present for the reversal of any particular reaction. So a complete reversal of a generic reaction which puts the species entirely outside of the normal generic habitat, is more likely than the slight modification necessary to put it into a near but only slightly different habitat in which it would be held by only a slight gradation of the general reaction.

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[9] Walker, see ref. 8, p. 33.

[10] Kennedy, "The Ecological Relationships of the Dragonflies of the Bass Islands of Lake Erie," *Ecology*, Vol. III., pp. 325–336, 1922.

At first thought, if it is easier to have a reversed reaction in the insect nervous system than to have a graduated reaction, one might think that the genus would fly apart as to any unity of environment, so that each species would have a habitat strikingly different from that of each other species, that there would be no such thing as a generic habitat. Observation shows that this is not so. Species are superimposed and habitats overlap in a most confusing manner and in any large genus there is usually an easily recognized generic type of habitat. So far not enough experimental work on behavior has been carried out to determine if the species of any one genus of insects are distributed by graduated reactions to one type of stimulus. However an analysis of some of the factors of the problem appear to indicate that such a distribution, for instance as the species of *Æshna*, show in a series of temperature gradations, may not be due to a slight difference in reaction to a specific stimulus, but may be merely an apparent series each species of which is held in its particular zone by some different positive or negative reaction to any one of a variety of stimuli..

An insect with incomplete metamorphosis passes through two stages, nymph and adult, in each of which it may pass through several physiological stages of development. The nymph has at least two, its feeding stage and its quiescent preëmergence stage. The imago has at least five stages, the teneral, the sexually mature stage, which can be divided into periods of hunger, periods of sexual lust, periods of egg-laying, and finally the stage of senility. The last need not be considered in a problem of species distribution as it is beyond and outside the genetic cycle. In each of these stages several stimuli control the individual, the reaction to anyone of which may be the factor that limits the species to its particular environment. If we consider only ten of the more easily recognized tropisms then in the six physiological stages enumerated above we have one hundred and twenty positive and negative possibilities for a reaction to an individual stimulus that might limit the species to a specific habitat. The problem is really much more complicated than this as many more reactions enter and all are more or less conditioned by morphological factors. So there is no difficulty at all in accounting for a great variety of factors, any one of which may limit the species to a rather narrow habitat.

In building the conception in the student's mind of the group of habitats occupied by all the species of a genus the mind automatically picks the most obvious habitat characteristic that is common to all the habitats under consideration. If this habitat characteristic, such as flying temperature for *Æshnas*, varies from habitat to habitat the mind automatically considers each species delicately adjusted to the exact degree of this variation in its specific habitat. This may not be true at all. And the factor which limits each species may not be the obvious one but some inconspicuous factor and it may be in each species in the genus a factor from a different category. It may be a temperature factor in one, a moisture factor in another, a chemical factor in a third species, etc. Thus graduated generic factors may largely be a compound figment of the mind of the observer.

THE INDIVIDUALITY OF THE GERM-NUCLEI DURING THE CLEAVAGE OF THE FERTILIZED EGG OF THE ROTIFER, *ASPLANCHNA INTERMEDIA*.[1]

BARBARA WIGGENHORN,

DAVID D. WHITNEY.

Recently while studying sections of the rotifer, *Asplanchna intermedia* (Hudson and Goss), double nuclei were discovered in the fertilized eggs while only one nucleus was seen in the parthenogenetic eggs. All of the cells in the early cleavage stages showed the two nuclei but in the later stages fewer and fewer cells contained two nuclei. It was decided to study the sections in an attempt to determine how late in the embryonic development the two nuclei remain distinct.

This species produces small parthenogenetic eggs developing into males, larger parthenogenetic eggs developing into females and large fertilized eggs which also develop into females. Both kinds of parthenogenetic eggs may be distinguished readily by their very thin covering membrane. It appears to be no thicker than the covering membrane of the blastomeres of an embryo. (Fig. 1, *A–G*). The covering of the fertilized egg is many times thicker and also shows a different structure than that of the parthenogenetic eggs. By noting the covering membranes alone one can distinguish these two kinds of eggs at a glance. (Fig. 1, *A*, *D*, *H*). No observer has ever found a parthenogenetic egg with a thick shell in this species.

When the male parthenogenetic egg is fertilized it becomes larger, develops this thicker characteristic shell and is popularly known as the winter egg. The origin of both of these eggs is identical but their mature appearance depends upon whether they are fertilized or not. They both produce two polar bodies while the female parthenogenetic egg only produces one polar body.

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[1] Studies from the Zoological Laboratory, The University of Nebraska, No. 142.

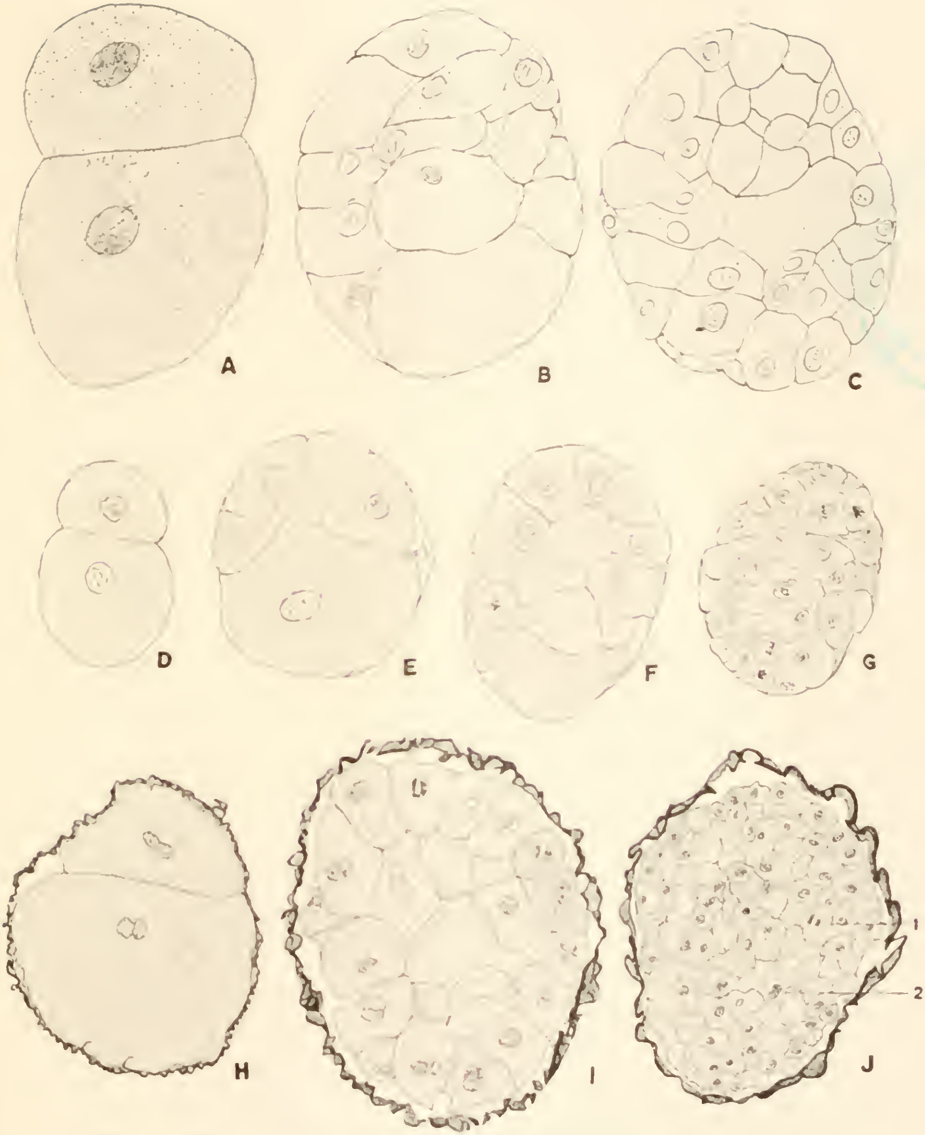

FIG. 1. *A–C*, sections of early embryos developing from parthenogenetic female-producing eggs showing one nucleus in the cells; *D–G*, sections of early embryos developing from parthenogenetic male-producing eggs showing one nucleus in the cells; *H–J*, sections from embryos developing from fertilized eggs showing two nuclei in some of the cells.

As this form of rotifer is oviviviparous and the females frequently carry from 1–10 developing embryos and young it was found practical to fix entire individuals in the killing fluids. Flemming's strong solution for about one hour was used. Then the material was bleached, dehydrated and imbedded in clove oil and paraffin. Large numbers of individuals were sectioned in masses, cutting sections 5 microns in thickness and the favorable sections studied. These animals were raised in great numbers in laboratory cultures in weak horse-manure solutions. The figures were outlined by means of a camera lucida with a magnification of about 430 diameters and the details filled in free hand.

As it is the male parthenogenetic egg that can be fertilized and changed into a larger and thicker shelled egg it is of some interest to compare the nuclei of the cells in the embryos developing from these eggs without fertilization and with fertilization. For a complete comparison the female parthenogenetic egg has been included.

Each cell of the embryo developing from both kinds of the parthenogenetic eggs contains only one nucleus regardless of whether it is in the 2-cell stage or in later stages of a larger number of cells (Fig. 1, *A–G*). Many hundreds of sectioned embryos have been observed in the various celled stages and not one has been found whose cells contained more than one nucleus.

A few somatic cells were found in which the chromosomes could be counted fairly accurately in the embryos developing from both kinds of the parthenogenetic eggs. The small male embryos developing from the male parthenogenetic egg showed the haploid number of about 25 and the larger female embryo developing from the female parthenogenetic egg showed the diploid number of about 51 chromosomes (Fig. 2, *A–B*). Although the chromosomes are very small and somewhat crowded together their exact number is reasonably certain. Tauson working on this same species has concluded the haploid number in the mature male parthenogenetic egg to be 24 and the diploid number of 48 in the female parthenogenetic egg.

In the early embryos developing from the fertilized eggs two distinct nuclei appear in each cell. These nuclei usually appear apposed to each other but clearly distinct (Fig. 1, *H*). In later stages only some of the cells show the two nuclei and as the embryo becomes older and is comprised of many more cells very few of the cells are found containing two nuclei, as for instance about the 250-cell stage only two such cells were found in a section (Fig. 1, *I–J*). Beyond that stage no cells have been found containing two nuclei.

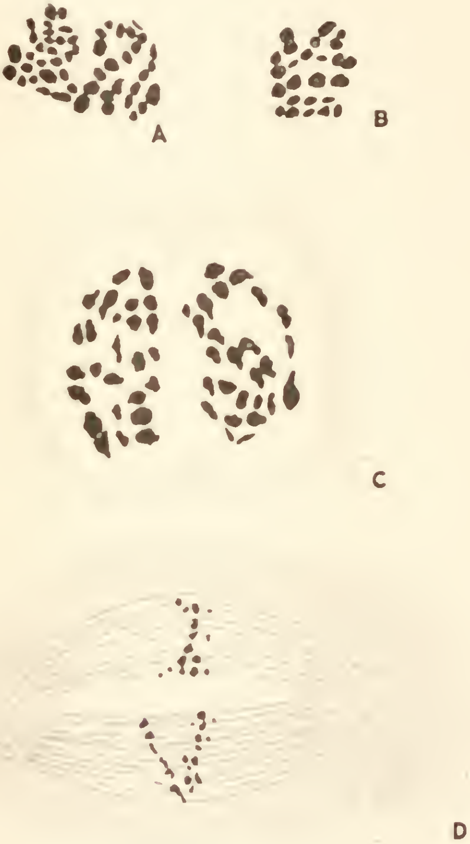

FIG. 2. *A*, somatic cell of a female embryo developing from a parthenogenetic egg showing 51 chromosomes; *B*, somatic cell of a male developing from a parthenogenetic egg showing 25 chromosomes; *C*, the two nuclei of a matured and fertilized egg showing 26 chromosomes in each nucleus; *D*, a double spindle from a somatic cell of an embryo developing from a fertilized egg. Drawings somewhat diagrammatic because of minute size of chromosomes.

Mitotic figures occur showing double spindles which seem to indicate that the two nuclei divided independently of each other in cell division (Fig. 2, *D*). Why this occurs only in the cells in the early stages of the embryo is not determined. Perhaps as the cells become more numerous and consequently smaller the size ratio between nucleus and cytoplasm is so altered that there is only room for one mitotic figure and consequently both nuclei are crowded into one fused nucleus.

A section was found of a fertilized egg in the one-cell stage in which the chromosomes could be counted fairly accurately. Each nucleus seemed to have 26 chromosomes. This fact clearly differentiates it from either of the parthenogenetic eggs. It also furnishes additional evidence to support the claim that the fertilized egg was originally the male parthenogenetic egg which has been entered by the sperm. The haploid number of 26 chromosomes is found in the mature male parthenogenetic egg and in each of the two nuclei of the fertilized egg. One of these nuclei in the fertilized egg is undoubtedly the egg nucleus and the other is from the sperm (Fig. 2, *C*).

Further observations of the sections of the fertilized eggs show that in the first cleavage each nucleus gives rise to two groups of chromosomes each of which passes separately to the daughter nuclei (Fig. 2, *D*). During the ensuing resting stage each germ nucleus is represented by a structurally distinct vesicle. Thus the separateness of the germ nuclei is maintained throughout the entire nuclear cycle. In mitosis there seem to be two spindles each with its distinct set of chromosomes which separate regardless of each other. Probably each group of chromosomes splits into halves, and a maternal and a paternal group go to each end of the double spindle, so that each daughter cell receives two sets of chromosomes around which separate walls are formed, and a maternal and a paternal vesicular nucleus appear.

The double nuclei have been found up as far as about the 250-cell stage. In one section of an embryo there were 103 visible cells and among these two had double nuclei (Fig. 1, *J* 1–2).

Two distinct nuclei in fertilized eggs and their developing embryos have been observed in other forms by various other workers. Mark observed this phenomenon in *Limax*, Van Beneden in *Ascaris*, Häcker in *Cyclops*, Conklin in *Crepidula*, Beard in *Raja batis*, Smith in *Cryptobranchus alleghenensis* and other workers have seen indications of it in other forms. No one has observed this in rotifers previously nor have the two nuclei been seen in as late stages as in this species of rotifer.

Summary: It has been found in *Asplanchna intermedia* that the germ nuclei in the fertilized egg do not fuse in the early cleavage stages, but each gives rise to two sets of about 26 chromosomes, one set of which pass into each of the daughter nuclei. Each nucleus is a structurally distinct vesicle.

In later cleavage stages the nuclei become fused so that in about the 250-cell embryo only a very few show the two nuclei in one cell.

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THE RELATION OF CARBON DIOXIDE TO SPONTANEOUS MOVEMENTS IN THE LARVÆ OF *OPSANUS TAU*.

HENRY C. TRACY.[1]

Spontaneous movements in embryos have been reported in the literature since the time of William Harvey (1651). Such movements have been observed in many groups of animals, and their occurrence is probably a universal phenomenon.[2]

More or less definite statements as to a relation between embryonic movements and respiratory conditions, have appeared several times in the literature.

Balfour ('76) says: "The band [of longitudinal striated muscles] developed at this stage [*H*] appears to be a special formation which has arisen through the action of natural selection, to enable the embryo to meet its respiratory requirements, by continually moving about, and so subjecting its body to fresh oxydizing influences; and as such affords an interesting example of an important structure acquired during and for embryonic life."

Ahlfeld ('05) showed the existence of rhythmic movements in the human fetus during the second half of pregnancy, manifested by rhythmic undulations of the maternal abdominal wall. These movements were near the rate of respiration in the newborn and were interpreted as preliminary respiratory movements.

Sarwey ('15) states that there is an increase in the strength and frequency of fœtal movements at the beginning of asphyxia and shortly before death.

Minkowski ('21) says: "On admet generalment que l'asphyxie de la mere mene a une augmentation des mouvements fœtaux, l'asphyxie directe du fœtus pourrait donc egalement le faire. Le manque d'oxygene agirait alors comme une excitation interieure provenant du sang, et determinerait ou favoriserait du moins les mouvements du fœtus."

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[1] Contribution from the laboratory of the United States Bureau of Fisheries, Woods Hole, Mass., and the Department of Anatomy, University of Kansas.

[2] References to the literature on these movements may be found in Preyer, '85; Munkowski, '21, and Wintrebert, '20.

Wintrebert ('14) states that in the trout the embryonic contractions are more frequent and lively in a medium containing CO_2 than in one containing oxygen. He considers ('20, pp. 450), these movements favorable to respiration, excretion, and circulation. He also states that lack of oxygen depresses the movement (in Selachians).[1]

Experimenting with cat fetuses eight to nine centimeters long, T. Graham Brown ('14) observed that rhythmic alternating movements of the limbs (interpreted as progression movements) arose spontaneously and could also be produced by asphyxia resulting from pressure on the umbilical cord.

In adult animals, the relation between the respiratory center and CO_2 has long been known, even though the exact mechanism is still in doubt. Stimulating effects of asphyxial conditions on blood pressure and motor mechanisms through the spinal cord have been reported (Mathison, '10 and '11).

A. D. Waller ('96) found an increased irritability in nerve fibers treated with CO_2, the effect varying with different concentrations. Roaf ('12) showed that the rate of branchial movements in fish showed a positive correlation with the H-ion concentration and with the concentration of CO_2; the appendages of barnacles showed a negative correlation to these conditions.

These observations and other well known physiological studies indicate that stimulation by metabolites occurs in many different varieties of contractile and transmissive mechanisms; it, therefore, may be expected that alteration of body fluids with respect to respiratory conditions may have some relation to the endogenous movements of embryos, and in fact it is possible that changes in such conditions may be the most important factors in the production of these movements.

The experiments reported in this paper were undertaken in order to test the effect of different concentrations of CO_2 in the sea water on the spontaneous movements of larvæ of *Opsanus tau*.

The yolk sac, with the network of capillaries over its surface, furnishes favorable conditions for an interchange of substances between the sea water and the blood of the larvæ and probably acts as a respiratory organ before the branchial mechanism becomes functional. It is, therefore, comparatively easy for purposes of experiment to change certain chemical characteristics of the body fluids of the larva by altering the character of the sea water around it.

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[1] So far as his descriptions go, the depression of movements which he observed appears to have taken place under asphyxial conditions and were probably due to the narcotic action of a considerable excess of CO_2 (Wintrebert, '20, pp. 324 and 328).

Opsanus tau (toad fish) is an inactive bottom fish which inhabits shallow, sheltered water. The eggs are found in such localities, fertilized and in the process of development, attached to the under-side of sticks, stones, tin cans, etc. The objects to which the eggs are attached may be brought into the laboratory where they develop in an apparently normal manner if furnished with a constant supply of fresh sea water. The eggs hatch in 3 or 4 weeks; the yolk sac, however, remains attached; about three weeks later, the larva has absorbed nearly all the yolk; it then becomes loosened from its attachment and swims free.

In this species the first observable movement is the heart beat which begins in specimens of 12 to 14 somites. The earliest stage at which muscular movements of the body were observed was in a specimen about 19 somites. Movements appear in the first few somites (2 to 4 or 5); the somites contract slowly and apparently simultaneously, causing a lateral bending of the anterior body region.

These movements take place singly and at irregular intervals, often with several minutes between and with no apparent relation between successive movements. Responses to external stimuli, however, do not take place until a much later period, in fact, not until after hatching. There seems, therefore, no question that these early movements are the result of changes in the internal conditions.

Soon, however, there appears a slight tendency for movements to appear in groups; this tendency increases until at the time of hatching, in addition to occasional single bendings of the body, right and left alternating coils of the body often take place very rapidly for a brief interval, producing a kind of vibratory or "fluttering" movement of the whole body. These "fluttering" movements become more and more predominant during the larval stage. When the free swimming period begins the "fluttering" movements of the body which alternate with irregular intervals of rest have now become regular undulating vibrations which cause short bursts of swimming movements of the fish through the water.

It is evident that the spontaneous movements of the embryo are gradually elaborated into the swimming movements of the free larva. Similar bursts of motion at the end of considerable intervals of rest on the bottom are characteristic of the adult throughout its sluggish existence. In the other teleosts which I have observed (*Fundulus* and *Tautogalabus*), the embryonic movements occur with much greater frequency and with only very brief intervals between; during development these movements become merged into the continuous type of movements characteristic of fishes of pelagic habits. It would appear that the activity habits of these animals are not widely different at any stage of their existence, and are determined by some internal physiological mechanism, the earliest expression of which is found in the spontaneous movements of the embryo.

Spontaneous movements of the mandible and branchial mechanism begin soon after hatching; they are, at first, slight and irregular, but gradually, in the course of about 5 days, they develop into the respiratory rhythm. Exteroceptive and proprioceptive reaction mechanisms do not respond to external stimuli at the time of hatching but gradually become functional during the larval period.

Preliminary experiments were carried out in the summer of 1922 at the Marine Biological Laboratory at Woods Hole.[1] Newly hatched larvæ were exposed to sea water from which all the gases were removed by passing hydrogen for an hour and a half through sea water which had been previously boiled.

Of 13 specimens, under these conditions, 11 showed a decrease in spontaneous movements. In another set of experiments 8 larvæ were exposed to sea water in which a number of *Fundulus* had been allowed to remain until they showed signs of asphyxiation; of these, 6 specimens showed a decided increase in spontaneous movements. In another experiment, 6 specimens were placed in KCN $n/1000$ in sea water. In all of these specimens, the movements were immediately stimulated to such an extent that they were nearly continuous during the first 15 minutes; they slowed down gradually, reaching about normal at the end of the first hour; during the next following 15 minutes, the movements ceased entirely. The results of these experiments show a close resemblance to reactions which would have been expected from a respiratory mechanism under similar conditions.

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[1] The research room at the Marine Biological Laboratory was supplied me from the Graduate Research Fund of the University of Kansas.

During the summer of 1923 at the laboratory of the United States Bureau of Fisheries,[1] at Woods Hole, I attempted to carry out more accurate experiments by subjecting *Opsanus* larvæ to sea water of known definite partial pressures of CO_2. For suggesting this method I gratefully acknowledge my indebtedness to Doctor Homer W. Smith. Details of the method have since been published (Smith and Clowes, '24).

METHODS.

The method of experiment consisted essentially in adding different percentages of HCL *n*/20 to the sea water in which the larvæ were placed. The changes in CO_2 partial pressure, and the H-ion concentration produced by this means are stated in the tables.

In applying this method, the following sources of error had to be taken into account:

(1) Variation in frequency and duration of the spontaneous movements in different individuals, and in the same individual at different times.

(2) Temperature changes.

(3) Stimulation by manipulation and handling of the embryo previous to the beginning of each experiment; previous observations had shown that manipulation of the yolk sac which results from handling of the larvæ, pressure of flowing water, etc., may cause an increased movement even though the larvæ do not respond to direct tactile stimulation.

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[1] In connection with laboratory arrangements in the conduct of these experiments I am greatly indebted to Dr. R. E. Coker, Director of the Laboratories of the Woods Hole Station of the U. S. Bureau of Fisheries.

(4) Stimulation of movements which might result from the shaking the bottle containing the specimens, after the addition of the acid to the sea water.

To control these sources of error, the following routine procedure was adopted. The larvæ were taken from the stock in the aquarium, scraped off from their attachment, and placed in a 250 cc. flask of fresh sea water at 25° C., and allowed to remain undisturbed from one half hour to an hour. The bottle containing the larvæ was then shaken a definite number of times (20 uniform back-and-forth movements). This procedure served as a control for the shaking after addition of the acid; several experiments seemed to show that stirring of the sea water in this manner had no appreciable effect on the movements of the larvæ, but nevertheless, at the beginning of each record of normal movements the bottle was shaken as described. The movements of the larvæ were then recorded for a period varying from 15 minutes to one half hour; this furnished the record of the movements under normal conditions of each individual at 25° C. At the end of this time, a little sea water was poured out of the bottle, the proper amount of HCL *n*/20 added and the contents of the bottle made up to exactly 250 cc. with sea water and the stopper inserted. These operations were carried out as rapidly as possible in order to prevent the escape of CO_2 into the air. The bottle was then stirred (as described above for the normal control) in order to secure as rapidly as possible a uniform CO_2 pressure and H-ion concentration throughout the water in the bottle. The movements were then recorded, usually for 30 minutes to one hour. At the close of this period, the acidulated sea water was poured off, and replaced with fresh sea water of the same temperature. The slight agitation of the larvæ which was unavoidable in pouring off the acidulated water is probably a sufficient control for the stirring at the beginning of the previous steps in the experiment. A record of the movements was then made.

In making the record, the time, character and extent of the movements were noted. A single coil to one side was taken as the unit. The number of separate movements can be recorded in this way with considerable accuracy; but in the case of movements following each other in rapid succession, as in the "fluttering" movements mentioned above, a somewhat arbitrary method is necessary. These "flutters" were assigned a value of 10, which seems about the minimum estimate of their value. At the free swimming stage these "flutters" sometimes continue several seconds which, of course, cause an extended swimming movement; a value of 10 for each second was given for such movements. The results thus arrived at are partly based on estimates and hence cannot be exact. But they will serve to give the general order of magnitude of the movements of the larvæ under different conditions. Most of these movements are undoubtedly greater than the minimum assigned value; hence the error favors a negative result. Since the method of estimation of numerical value was the same for the same larva under both control and experimental conditions, errors due to the method of recording tend to neutralize each other.

The records of each experiment were afterward transferred to chart records by recording the total numerical value of the movements for each minute on cross-section paper. The records of all specimens for each concentration of CO_2 were then averaged for each minute; then, in order to smooth the curves somewhat, averages for each two minutes were taken and recorded as shown on the charts accompanying this paper.

Observations were made at two periods of the larval development:

(1) Within the first five or six days after hatching and before external respiration had begun (larvæ about 6 or 7 mm. in length). At this stage there is no response to light, rotation, jar, or vibrations and little or none to tactile stimuli.

(2) At the end of the larval period when the yolk sac is nearly absorbed, and the larvæ are on the point of swimming free (18–20 mm. in length). All the reaction mechanisms, as far as known, are now functionally developed.

The behavior of the respiratory mechanism in the free swimming larvæ (second period) was observed under the different conditions of CO_2 and the record superimposed on the charts recording the body movements. The time in seconds necessary for the completion of 10 respiratory movements was taken with the stop watch for each individual (whenever possible the average of 3 observations for each minute), and the average of all the individuals for each concentration of CO_2 was indicated on the charts for each two minutes by the number of spaces above the base line.

RESULTS.

In the first stage 3 or 4 specimens can be studied at the same time in one experiment; but in the free swimming stage only one specimen can be recorded at a time. The breeding period of this species is brief, hence, the number of individual records obtainable is not as large as is desirable, but when the averages are brought together the conclusions stated below seem justified. But the number is insufficient to give a smooth contour to a curve indicating the effect of any given concentration with respect to the time of exposure or to state in an exact manner the effect of different concentrations.

The chart records indicate the following relation between changes in the partial pressure of CO_2 and the body movements and respiratory rate.

A. Body movements.

1. The movements in each group show an increase during the first few minutes following an increase in the concentration of CO_2.
2. When the specimens in the water containing the higher concentration of CO_2 are returned to normal sea water, the movements in each group are depressed considerably below the normal. This depression is very pronounced during the first 10 minutes and gradually approaches the normal during the 30 minute period.
3. The intensity of the reaction varies with the increase in CO_2, up to the middle range of concentration; above this the intensity of stimulation decreases with the increase of CO_2 concentration.
4. The reaction to increased CO_2 is less intense but of greater duration in the earlier than in the later stage. On returning to normal sea water the depression of movement is less in the earlier than in the later stage and recovery is slower.

The different effects in the two stages are not easy to explain.

They might possibly be due to the presence of the large yolk sac in the younger stage. The mass of the larva at hatching is considerably less than that of the yolk; the yolk then must act as a "buffer" and the increased CO_2 is "soaked up" from the blood by the yolk substance inside the vitelline net work nearly as rapidly as the blood absorbs it from the water outside. The increase in concentration in the body fluids of the larvæ is, therefore, relatively slight at first and increases slowly until the yolk substance approaches equilibrium with the sea water relative to the CO_2 concentration. It is only in the high concentrations that this can take place during the first 10 minute period. In returning to normal sea water the blood begins to absorb the CO_2 back from the yolk and hence the depression period is pronounced.

At the beginning of the free swimming stage the amount of yolk remaining is very slight, and in the gills active interchange of gaseous substance is taking place directly between the sea water and the body fluids of the larva; the effect of increasing the concentration of CO_2 in the sea water is, therefore, almost instantaneous and hence the stimulation rapidly reaches its maximum, followed quickly by the depression due to effects of excessive CO_2. The recovery in the normal sea water is rapid on account of the rapid attainment of equilibrium between the inside and outside of the larva through the branchial system.

It is, however, difficult to understand why it should require so long a time for the yolk substances to take up the CO_2. According to the combined record of the newly hatched larvæ (Chart XII.) the heightened reaction lasts about 25 minutes, but only 10 minutes in the free swimming (Chart XIII.).

There are, of course, other differences between the two stages. In the first there is a relative preponderance of undifferentiated tissue, as compared with the later stage. The chemical relations of young tissue must be different from older. The larvæ will endure asphyxial conditions better than the adult (see p. 19). Anaërobic respiration is known in the young of other forms. Susceptibility to strychnine is less in young than in adults (Schwartze, '22). Apparently, young tissue has greater adjustment capacities to factors affecting a fundamental activity like metabolism than older tissue.

It must be stated that there are a few aberrant specimens which fail to react in the usual manner. The number and incidence of these are shown in the tables. Some of these exceptions are only apparent, since most specimens show movement either while the bottle containing them is being shaken or in the interval (a minute or less) immediately afterward, before the record can begin. These movements, of course, do not appear in the record. Also, there seem to be more aberrant specimens in the higher concentrations because of the stronger narcotic action of CO_2 and its more rapid onset. Some are perhaps pathological in spite of careful effort to select only healthy specimens; there is a large mortality under laboratory conditions, perhaps 50 per cent. during the larval period. Most of these exceptional specimens are probably thus explained; whether all can thus be accounted for, is uncertain; there may be physiological conditions in which an increased CO_2 may produce a depression immediately instead of stimulation.

B. Rate of Respiratory Movements.

In the lower ranges of concentration of CO_2, the respiratory rate is faster during the period of exposure (Charts VI., VII., VIII.). The middle ranges of CO_2 concentration (Charts IX. and X.) appear to result in a balance between the stimulating and depressant effects. In the higher ranges (Chart XI.) after a short period of stimulation, the rate falls considerably below the normal. In the extreme concentrations (addition of 3.5 per cent.–4 per cent. acid to the sea water—Chart XIV.) the respiration becomes irregular, then of the Cheyne-Stokes type; after a time it gradually improves and becomes regular, though proceding at a somewhat slower rate than in the normal. Reuss ('10) has shown the stimulating effect on the respiratory rate of moderate concentration of CO_2 in adult fish and the depressant effect of higher concentrations. There is, therefore, a tendency for these higher concentrations to break down the respiratory system; the capacity for recovery is probably dependent on some secondary reserve mechanism. This capacity for recovery seems not to be possessed by the spontaneous movements.

On return to normal sea water, the effect of the lower concentrations (Charts VI., VII.) is the same on the respiratory rate as on the movements, that is, a depression below normal;

in the case of the higher concentrations, the respiratory rate rises above the normal on return to normal sea water.

Obviously there is a close similarity between the effect of CO_2 on the rate of movements of the body and rate of respiratory movements in the lower concentrations; in the higher concentrations the respiratory mechanism has a compensatory capacity both during exposure and in recovery afterward, which is not possessed by the motility mechanism. Apparently, then, the regulation of respiration and motility at ordinary concentrations of CO_2 takes place through similar mechanisms; in high concentrations, the respiratory system can bring into play secondary reserve mechanisms, which appears to be beyond the capacity of the system controlling body movements.

The resistance of these larvæ to asphyxiating conditions is very great. One of these larvæ was left over night in a bottle containing the highest concentration of CO_2; the next day the specimen lay motionless on the bottom of the bottle with the respiratory rate about the same as shown on the preceding day; on putting the specimen into fresh sea water the respiratory rate quickly jumped above the normal, the body movements approached the normal in 12 to 15 minutes. Larvæ placed in water from asphyxiated *Fundulus* (which causes acute symptoms in half grown adult toad fish) apparently suffered no inconvenience; in boiled sea water, through which hydrogen had been bubbled for one and a half hours, the early larvæ show no effects during the first hour and a half (aside from reduction of body movements) although oxygen was entirely absent. Evidently anaërobic respiration is possible for a considerable time at least, in these embryos.

Stockard ('21, p. 173) indicated the probability that double and abnormal embryos in fishes may be produced by asphyxial conditions at certain stages. In the toad fish I have found only one double larva, although in the course of several seasons I have examined several thousand specimens. This high degree of resistance to asphyxial conditions is no doubt correlated with the crowded stagnant condition of the water in which this species passes its embryonic and larval life.

That there is some definite relation between spontaneous movements and respiratory movements is shown by the reduction in the rate of respiratory movements which seems always to exist immediately after each spontaneous movement (Chart XV.). At the end of each movement the branchial apparatus is motionless for a few seconds; it begins activity very slowly, and gradually resumes the normal rate in 10 to 15 seconds. This phenomenon may be due to acapnia induced by the rush of water through the gills while the larva is in motion; furthermore, if it is the production of metabolites which stimulates the body movements, the same substances would probably increase the volume of blood flowing through the branchial vessels and thus favor the washing out of CO_2 from the blood; a brief inactivity of the respiratory mechanism then ensues, due to the lack of stimulation.

Speculation as to the details of the mechanism by which embryonic movements are stimulated and depressed under the various conditions described in this paper seems premature until the relation between specific effect of CO_2 and variations in the H-ion concentration has been experimentally determined. Experiments with KCN, with different concentrations of CO_2, and with CO_2 removed, indicate that essentially similar mechanisms regulate respiratory and body movements, except for some accessory reserve system which exists in the case of respiratory movements.

The relation of different concentrations of CO_2 to the amount of motility must be a significant factor in determining the migrations and habitat of fishes. Shelford, Wells and Powers, (Shelford, '23) in their work on the relation of H-ion gradients to the movement of fishes apparently did not study the mechanism of the reactions. The results which these writers report are perhaps due to the direct effects of H-ion changes in the body fluids on the primitive neuro-muscular system, or, in other words, to variations in the rate of the endogenous, "spontaneous" movements conditioned by alterations in the amount of CO_2 or H-ions in the surrounding medium. According to this view, the optimum H-ion concentration is automatically determined by the effect of different parts of the acidity gradient on the endogenous movements, and not to any "choice" or "selection" on the part of the fish.

A question of broader significance is raised by these experiments since the results suggest that endogenous movements may be stimulated by variations, local as well as general, in the concentration level of metabolites which result from differences in the metabolism rate in different regions of the embryo. Jacobs ('22, p. 25) has made a similar suggestion regarding the production of pseudopods in *Amœba*.

SUMMARY AND CONCLUSIONS.

1. Newly hatched and free swimming larvæ of toad fish (*Opsanus tau*) were subjected to different concentrations of CO_2 produced by additions of different percentages of HCL *n*/20 to sea water.

2. Increased concentration of CO_2 is followed by an increase in the endogenous (spontaneous) body movements in both stages; in newly hatched larvæ the reaction of CO_2 is less intense but of greater duration (average about 25 minutes) than in free swimming larvæ (average about 10 minutes).

3. On return to normal sea water from the higher concentration of CO_2, the frequency of the body movements is depressed below the normal; the depression is less in the newly hatched larvæ than in the free swimmer and the recovery is slower.

4. In the lower ranges of CO_2 concentration, the body movements (in both stages) and the rate of respiratory movements (free swimmers) vary with the increase in CO_2; in the higher ranges the body movements, after a period of stimulation, remain depressed far below the normal, while the respiratory movements, after a brief stimulation followed by depression and irregular rythm, recover and proceed regularly at a little below the normal rate.

5. On return to normal sea water, the body movements for all concentrations of CO_2 remain depressed (about 30 minutes for the free swimmers, longer for the newly hatched larvæ); the rate of respiratory movements is below the normal for the lower concentrations, but is increased above the normal on return from higher concentrations.

6. Respiratory movements and spontaneous body movements react similarly to the lower concentrations of CO_2 and hence their regulation probably takes place through a similar mechanism; at the higher concentrations, the respiratory system

appears to bring into play a secondary reserve mechanism which gives it a compensatory capacity not possessed by the neuromuscular system through which body movements are produced.

7. Toad fish larvæ are much more resistant to asphyxial conditions than adults.

8. It is suggested that the migration of fishes in a H-ion gradient is probably conditioned by the effect of acid substances on the endogenous body movements.

9. It is suggested that stimulation by variation in the concentration level of metabolites produced inside the body may be the source of endogenous (spontaneous) movements.

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EXPLANATION OF TABLES AND CHARTS.

Charts I. to V. are records of specimens just hatched, before the beginning of respiration.

Charts VI. to XI. are records of specimens at the beginning of the free swimming stage.

Chart XII. is the combined record of the movements of the just hatched larvæ under all concentrations of CO_2.

Chart XIII. is the combined record of the movements and respiration rate of the free swimming larvae under all concentrations of CO_2.

Two minute spaces are marked off on the horizontal base line: height of the line indicates the average amount of movement (estimated as described on p. 10) made in each minute of the two-minute period by the specimens in each concentration of CO_2. The average amount of movement each minute under normal conditions for the time observed (15 minutes to 1 hour) is indicated by the arrow at the beginning of each chart, marked A. N. M. The arrow marked A. M. R. indicates the average movement per minute when the larvæ are returned from the acidulated water back to the normal.

In Charts VI.–XI. and XIII. the respiration record of the free swimmers is superimposed on the movement record (respiration not being established in the just hatched larvæ). The height of the short horizontal line in each two minute space indicates the number of seconds (usually the average of three observations in each minute) taken for 10 respiratory movements (average for all the specimens of each concentration of CO_2). Hence a drop in the line means increased respiratory rate, and vice versa. The observed normal rate (average for each minute) is indicated at the beginning of each chart by an arrow marked A. N. R.

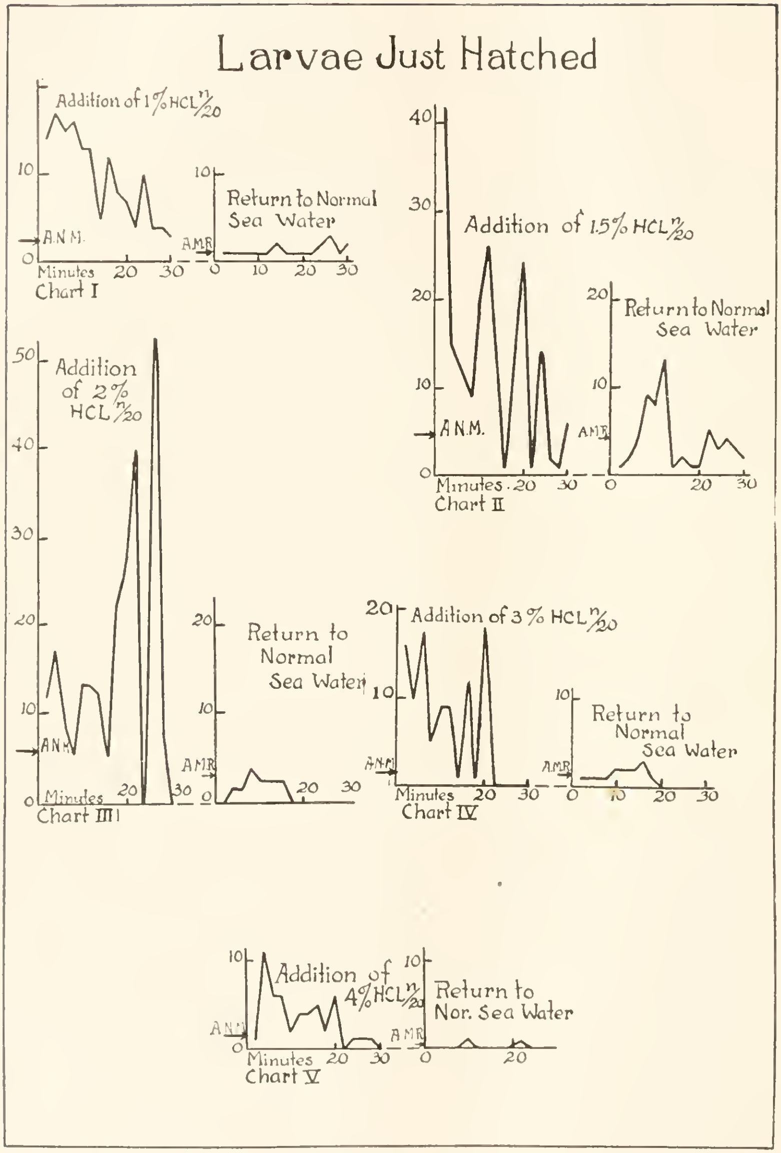
The arrow marked A. R. R. indicates the number of seconds required for 10 respiratory movements when returned to normal sea water from the acidulated water.

Chart XIV. shows the influence of high concentrations of CO_2 on the respiratory rate in two individuals (*A* and *B*).

Chart XV. shows the fall of the respiratory rate which is observed at the end of each spontaneous movement.

On Tables I. and II. the average number of movements (that is, the number of separate movements which took place regardless of extent and character) is given for each 10-minute period under different conditions.

In Tables III. and IV. is given the average numerical value (that is, the amount of movement, using the coil to one side for unity) for each 10-minute period under different conditions.

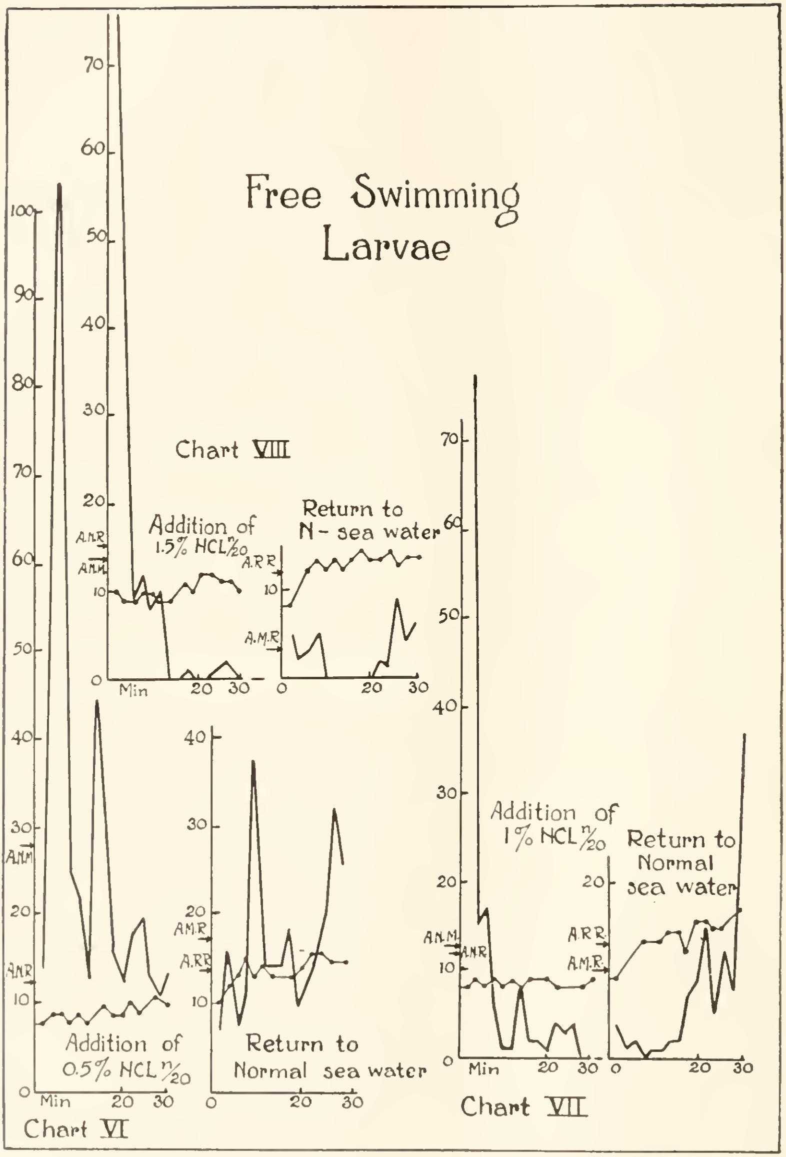

Larvae Just Hatched
Addition of 1% HCL n/20
Return to Normal Sea Water
A.N.M.
AMR
Minutes 20 30
0 10 20 30
Chart I
Addition of 1.5% HCL n/20
Return to Normal Sea Water
A.N.M.
AMR
Minutes 20 30
Chart II
Addition of 2% HCL n/20
Return to Normal Sea Water
ANM
AMR
Minutes 20 30
Chart III
Addition of 3% HCL n/20
Return to Normal Sea Water
ANM
AMR
Minutes 20 30
Chart IV
Addition of 4% HCL n/20
Return to Nor. Sea Water
ANM
AMR
Minutes 20 30
Chart V

CHARTS I–V.

TABLE I.

NUMBER OF MOVEMENTS IN 10 MINUTE PERIODS OF NEWLY HATCHED LARVÆ UNDER DIFFERENT CONCENTRATIONS OF CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13
% HCl $n/20$.	Partial Pressure CO_2.	P_H	Number of Specimens	Average Number of Normal Movements in 10 Minutes.	Average Number of Movements Each 10 Minutes under Increased CO_2.				Average Number of Movements per 10 Minutes when Returned to Normal.			
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	Number of Specimens not Stimulated.	Number of Specimens.	1st 10 Minutes.	2d 10 Minutes.	Number of Specimens Aberrant.
1	.010	7.0	13	6	9.6	4.7	5.4	4	8	1.4	3	2
1.5	.0185	6.64	4	5.15	10.3	3.5	4.5	1	4	6.3	7.7	2
2	.025	6.42	8	13.2	13.5	6.7	4	3	6	9	17.3	2
3	.038	5.96	9	11.4	11.4	7.5	0.7	4	9	10.2	7.6	2
4	.052	5.00	4	3.4	9.5	1.5	1.5	0	4	0.25	0	1
Averages and totals.......			38	8.4	10.9	5.3	3.5	12	31	5.4	7.3	9

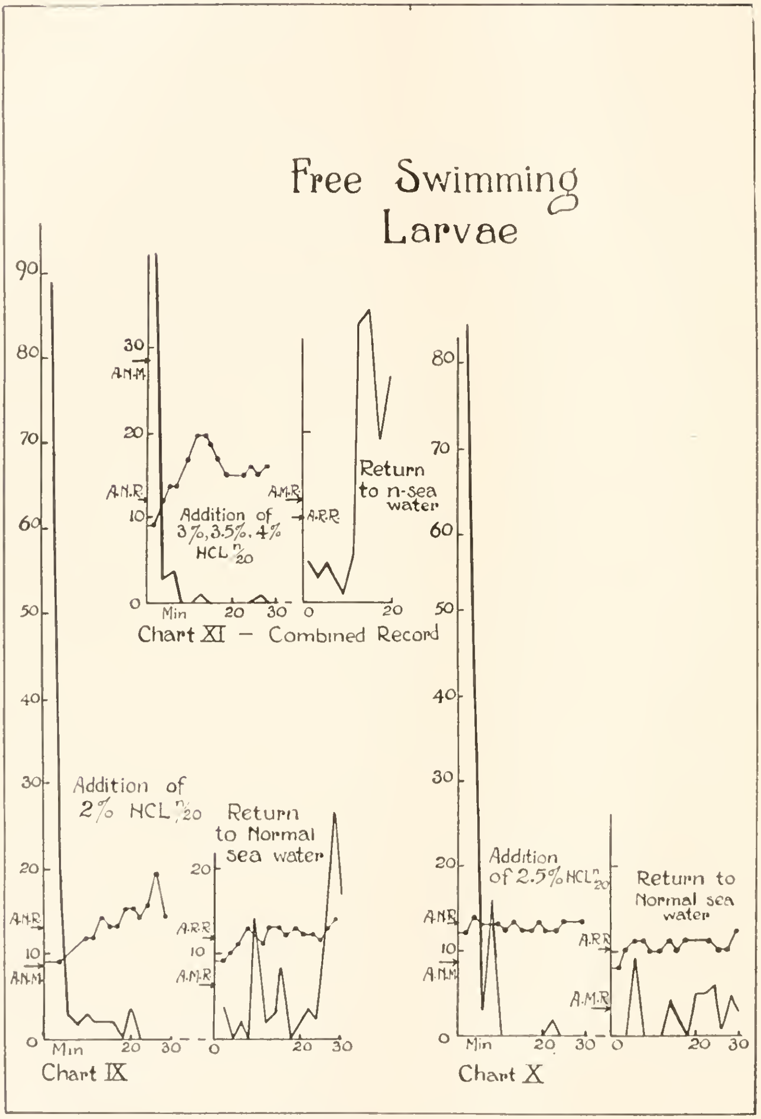


CHARTS VI–VIII.

TABLE II.

NUMBER OF MOVEMENTS IN 10 MINUTE PERIODS OF FREE SWIMMING LARVÆ UNDER DIFFERENT CONCENTRATIONS OF CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13
% HCl $n/20$.	Partial Pressure CO_2	P_H	Number of Specimens	Average Number of Normal Movements in 10 Minutes.	Average Movements of Each 10 Minutes with Increased CO_2.			Number of Specimens not Stimulated.	Average Movements per 10 Minutes when Returned to Normal.			Number of Specimens Aberrant.
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.		1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	
0.5	.005	7.5	3	21	46	17.7	22.3	0	15	15	26.5	0
1.0	.010	7.0	3	11.1	15.3	3.3	2.5	2	3.3	11	25.7	0
1.5	.0185	6.64	3	14.3	24.3	3.3	1.7	1	3.3	0.3	9.3	0
2.0	.025	6.42	4	14.2	8.2	4.5	1.8	2	4.0	2.5	12	0
2.5	.0315	6.2	2	10.3	10.0	0	0	1	3	3	6.5	
3.0	.038	5.96	1	20	6	0	0	1	15	22	—	0
3.5	.045	5.60	1	1.3	6	0	0	0	2	1	22	1
4.0	.052	5.00	2	27.5	2.5	0	0.5	2	25	3	10	0
Averages and totals.......			19	15.4	17.2	4.6	5.2	9	8.1	6.5	16	1

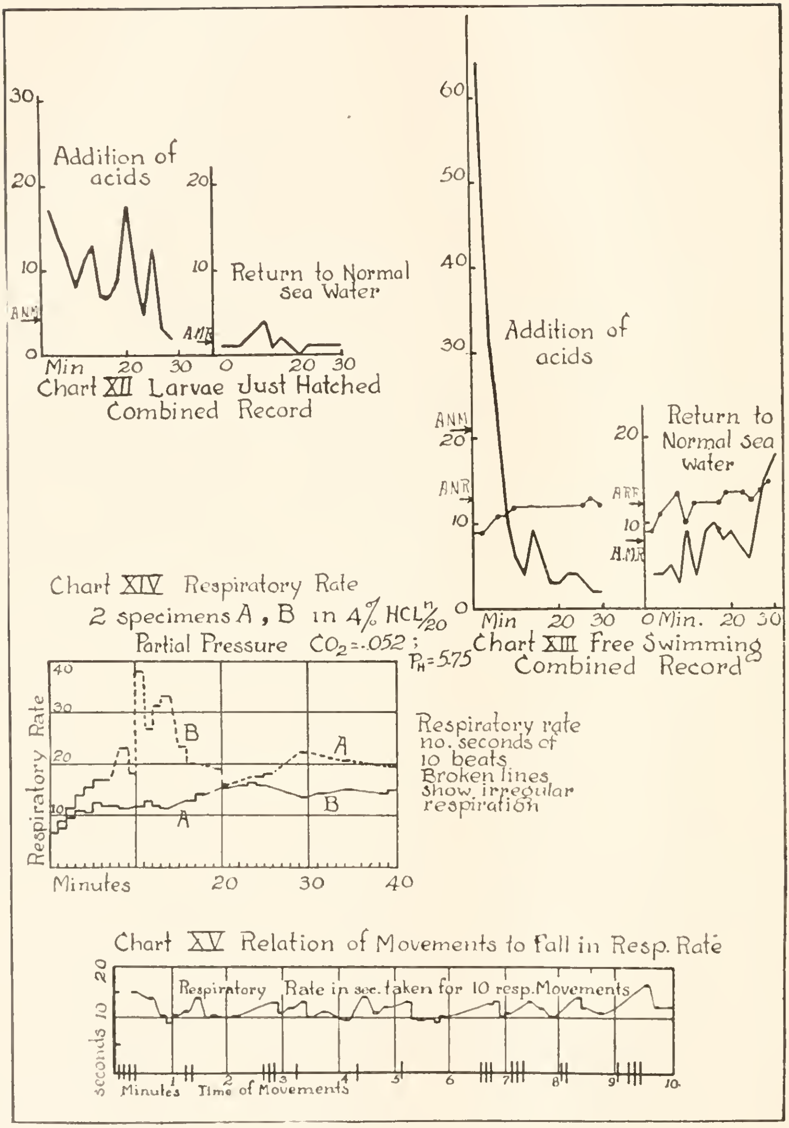

Free Swimming Larvae
Chart XI — Combined Record
30
A.N.M.
20
A.N.R.
10
0
Min
20
30
Addition of 3%, 3.5%, 4% HCL n/20
A.M.R.
A.R.R.
Return to n-sea water
0
20
90
80
70
60
50
40
30
20
A.N.R.
10
A.N.M.
0
Min
20
30
Chart IX
Addition of 2% HCL n/20
Return to Normal sea water
20
A.R.R.
10
A.M.R.
0
20
30
80
70
60
50
40
30
20
A.N.R.
10
A.N.M.
0
Min
20
30
Chart X
Addition of 2.5% HCL n/20
Return to Normal sea water
A.R.R.
A.M.R.
0
20
30

CHARTS IX–XI.

TABLE III.

NUMERICAL VALUE IN 10 MINUTE PERIODS OF NEWLY HATCHED LARVÆ UNDER DIFFERENT CONCENTRATIONS OF CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
% HCl n/20.	Partial Pressure CO_2.	P_H.	Number of Specimens.	Average Number of Normal Movements in 10 Minutes.	Average Numerical Value of Each 10 Minutes under Increased CO_2.					Average Numerical Value per 10 Minutes when Returned to Normal.			Number of Specimens Aberrant.
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	No. Specimens not Stimulated.	Number of Specimens.	1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	
1	.010	7.0	13	41.6	154.7	103.2	51.2	2	8	5.9	6.5	18.7	2
1.5	.0185	6.64	4	50.2	193	148	48.2	1	4	48.5	49.5	51.2	1
2	.025	6.42	8	68.4	112.4	53	—	4	6	22	15.1	—	0
3	.038	5.96	9	24.7	113.1	39.4	—	1	9	11.1	22.5	23.5	0
4	.052	5.00	4	24.5	42.2	42.2	6.7	1	4	0.3	0.0	0.8	1
Averages and totals.....			38	42.3	128.1	75.8	42.1	9	31	15.3	17.5	22.8	4



CHARTS XII–XV.

TABLE IV.

NUMERICAL VALUE IN 10 MINUTE PERIODS OF FREE SWIMMING LARVÆ UNDER DIFFERENT CONCENTRATIONS OF CO_2.

1	2	3	4	5	6	7	8	9	10	11	12	13
% of HCl $n/20$.	Partial Pressure CO_2.	P_H.	Number of Specimens.	Average Number of Normal Movements in 10 Minutes.	Average Numerical Value of Each 10 Minutes under Increased CO_2.				Average Numerical Value Each 10 Minutes when Returned to Normal.			Number of Specimens Aberrant.
					1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	Number of Specimens not Stimulated.	1st 10 Minutes.	2d 10 Minutes.	3d 10 Minutes.	
0.5	.005	7.5	3	287.4	497	219.6	195.3	1	162.3	130.6	156	
1.0	.010	7.0	3	247	249	21	27		21	47	168.0	1
1.5	.018	6.64	3	137.3	337	4.6	6.3	1	25.3	0.6	63.6	
2.0	.025	6.42	3	157.8	196.2	16.2	2.7	2	48	20.7	125.3	
2.5	.0315	6.2	3	93	267.3	3.3	0.3	1	20	23.3	53.5	
3.0	.038	5.96	1	245	43	0.0	0.0	1	107	18.8	0.0	
3.5	.045	5.60	1	8	60	0.0	0.0		20	20.3	0.0	1
4.0	.052	5.00	2	503	117.5	0.0	2.0	1	18	0.0	0.0	
Averages and totals.......			19	211.9	261.9	41.7	36.2	7	52.2	37.1	89.5	2

THE RELATION OF BODY TO ENVIRONMENTAL TEMPERATURES IN TURTLES, *CHRYSEMYS MARGINATA* BELLI (GRAY) AND *CHELYDRA SERPENTINA* (LINN.).

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Iowa State College, Ames.

Although a considerable number of workers have recorded their observations on the relation of body temperature to that of the environment of different so-called cold-blooded animals, only a comparatively few of these refer especially to the reptiles, as a group, or to the turtles in particular. With but the exceptions indicated below, all the records from the early workers were based upon a limited number of individuals taken for the most part at random within narrow limits of normal but slight environmental changes. It has thus become quite generally assumed by biologists that turtles together with other cold-blooded animals approximate the temperature of their surroundings. In view of the fact that turtles as a representative group of the reptiles have an unique phylogenetic position, spanning the gap as it were between the warm-blooded birds on the one hand, and the cold-blooded amphibians on the other, it was thought that a study of their body-temperature changes when followed through high and low critical temperatures as well as the ordinary non-critical ranges, might yield interesting data.

The earliest observation of the body temperature in turtles is probably recorded by Walbaum (1), in 1782. He found the temperatures differed only one or two degrees from that of its environment and fluctuated with it. Following Walbaum a considerable number of workers made similar observations during the first half of the nineteenth century. Milne-Edwards (2) in 1863, after giving a critical review of the work done on these forms by Czermach (3), Murray (4), Tiedemann (5), Davy, J. (6), and Valenciennes (7), concluded that the body temperatures varied but little from that of the environment wherein the limits did not exceed 4 degrees C. It is of interest that the same author noted the observations of Valenciennes (7) which were later substantiated by Sclater (8), that the female python when coiled about the eggs during incubation, maintained a body temperature considerably above that of the surrounding air. Sclater found on comparing the body temperature of the incubating female with that of the male, that during the height of incubation the difference was as much as 10 degrees C., and that she was some 20 degrees C. above the surrounding air. Whether this marked increased in heat production was brought about by bringing into play a thermal regulatory mechanism, or was due to other causes was not stated and has not yet been learned. Similar observations were reported in 1881 by Forbes (9), with somewhat less conspicuous differences. The greatest difference of the air and the surface coils of the snake was about 6 degrees in the male, and about 9 degrees C., in the female. It was noted in this study that the female took no food and was comparatively inactive for weeks before and during incubation.

In 1903, Martin (10) working with the respiratory exchanges in Monotremes and Marsupials included some observations on the blue-tongue lizards (*Cyclodus gigas*). These, five in number, he carried through changes in temperatures varying from 5 to 39 degrees C., within a calorimeter. At room temperature they were comparatively active but became quite inactive at 5 degrees. On warming they increased in activity up to until about 30 degrees and above this their activity diminished. The body fluctuations accompanying these changes were noted. Throughout the middle ranges (10–35 degrees C.) the body temperature was a function of the environment but the CO_2 production was fairly constant. At the extremes (below 10 and above 35) as shown in his plots, Fig. 3, sharp breaks occur, with approximation to Van't Hoff's law. Notwithstanding that he kept the animals in an environment of between 39 and 40 degrees C., for over two hours he was unable to get their mean temperature above 38.5. In their work on certain of the cold-blooded animals Rogers and Lewis (10) followed the body temperature fluctuations in only two representatives of the vertebrates, the fishes (goldfish) and the amphibians (salamanders) but concluded that neither of these forms possessed mechanisms for either heat production or heat loss.

Experimental.—Turtles were collected in the vicinity of Lakeside Laboratory, Lake Okoboji, Iowa. The painted variety was unusually abundant in July and August in the shallow cove at Miller's Bay. The snappers were comparatively rare and could not be obtained in considerable numbers. Most of these were taken from Hottes' and Marble Lakes, west of Spirit Lake, while others were found along the shores of Little Sioux river and in the neighborhood of Hanging Bog. Suitable specimens of various sizes were placed in a live box as caught, and from there were taken into the laboratory as needed.

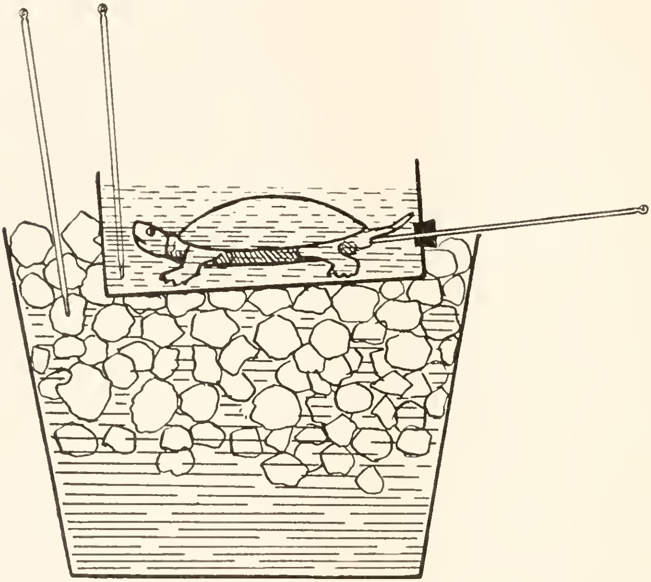

FIG. 1. Diagram of relationships of the apparatus used in cooling experiments. The animals were made fast by clamp in the smaller container surrounded by water, and this subjected to an ice bath. Temperatures were read from the three thermometers shown.

Individuals were weighed and mounted in such a way that their temperature changes might be recorded. Those placed in air as a surrounding medium were fixed to a board by heavy rubber bands slipped about the carapace and plastron and spaced so that the animal was unable to release itself by kicking.

The board was then supported at either end high enough above the table that the animal could not get sufficient traction to crawl. In this position the tail could be tied back and the thermometer inserted in the rectum.

Experiments with water as the surrounding medium were more difficult to carry out, and involved the construction of special containers which could be adapted to different sizes of individuals (Fig. 1). Shallow tin dishes of convenient sizes were found to fit the purpose, allowance being made so that a stoppered thermometer could be supported at the side. In mounting, the thermometer could be slipped into the rectum, and the animal made fast by adjustable wire clamps. Mounted in this way the specimen could be subjected to an ice mixture and its changes in body temperature as well as those of the environment be recorded.

Although some slight variations in the experimental technique were followed for checking purposes, in general the procedure in all cases involved the tabulating of the date of the experiment and the time intervals of the observations, the room, as well as the immediate environmental and the subject's temperature, the subject's number and its weight, and remarks on the behavior and stages of activities of the animal, under the conditions of the experiment. Some forty experiments exclusive of several preliminary observations, furnish data for this report, these being apportioned about equally between the two forms of turtles studied. For convenience, the discussion of results are grouped under the following captions:

Body Temperature Fluctuations in the Non-critical Ranges.—In a series of thirteen experiments, the diurnal and nocturnal fluctuations in body temperature were taken on individuals of the painted and snapping varieties, both in air and water. A typical chart of observations taken at intervals over a twelve-hour period from a painted turtle mounted in air, weighing 485 grams is given in Table I.

In general it is noted that the body temperature lags from one to three degrees as the temperature rises, and remains slightly above as it is lowered, being slightly effected by the state of the activity of the animal. The same fluctuations are apparent during the nocturnal intervals. Similar observations were made upon the snapping turtles in air and in water but since comparable results were obtained through these ranges it is unnecessary to cite typical protocols.

TABLE I.

AUGUST 1, 1924. OBSERVATIONS SHOWING THE TYPICAL FLUCTUATIONS OF BODY TEMPERATURES WITH ENVIRONMENTAL TEMPERATURES IN THE PAINTED TURTLE.

Date.	Time.	Room Temp.		Subj. Temp.		Diff.		Activity.
Aug. 1	8.00 A.M.	63° F.	17.5° C.	61° F.	16.0° C.	−2.0° F.	−1.0° C.	Quiet
	9.00	63	17.5	61.5	16.3	1.5	−0.8	"
	9.30	63	17.5	63	17.5	0	0	Active
	10.00	64	18	64	18	0	0	"
	10.30	65	18.5	64	18	−1.0	−0.5	"
	11.00	67	19.5	65	18.5	−2.0	−1.0	Quiet
	11.30	69	20.5	65	18.5	−3.0	−2.0	"
	12.00	70	21.0	68	20	−2.0	−1.0	Active
	12.30 P.M.	71	21.5	69	20.5	−2.0	−1.0	"
	1.30	70.5	21.3	70	21.0	0.5	0	"
	2.00	70	21.0	70	21.0	0	0	"
	3.00	69	20.5	69.5	20.5	0.5	0	"
	4.00	67	19.5	69	20.5	2.0	1	"
	7.00	67	19.5	69	20.5	2.0	1	"
	8.00	66.5	19.5	68	20.0	1.5	0.5	Quiet

Body Temperature Fluctuations on Cooling.—In these experiments *twelve* specimens of the painted variety ranging in weights from 265 to 712 grams, and ten snappers ranging from 152 to 1725 grams were used. Some of the representatives of each group were subjected to rapid environmental drops while in others the drop was made more slowly. This procedure was followed because in the preliminary observations it was noted that differences in rate of cooling seemed to effect slightly the body temperature fluctuations. In some cases the same individuals were rechecked on different days following the same procedure, and while slight divergences appeared in the records, the same general tendencies were apparent. Illustrating the chief points concerned with the rapid cooling of the painted variety data are given in Table II., which is taken from an experiment performed on Aug. 1, on a medium-sized (360 gms.) turtle, and which is fairly typical of them all.

This particular experiment began at nine o'clock in the morning and extended over five hours, the rapid drop in environmental temperature occurring during the first half hour. Although the

TABLE II.

PAINTED TURTLE, ENVIRONMENTAL AND BODY TEMPERATURES.

RAPID COOLING.

Time.	Room Temp.		Sub. Temp.		Env. Temp. H_2O.		Diff.		Activity.
	F.	C.	F.	C.	F.	C.	F.	C.	
9.00 A.M.	63°	17.5°	63°	17.5°	60°	15.5°	3°	2°	Quite active
9.30	63	17.5	62	16.8	36	3.5	26	13.3	Slow move
10.00	64	18	62	16.8	35	3	25	13	" "
10.30	65	18.5	61.5	16.3	34.5	2.5	27	12.8	" "
11.00	67	19.5	61	16	34	2	27	14.0	Quiet
11.30	69	20.5	58	14.5	34	2	24	12.5	"
12.00	70	21.0	56.6	13.3	33	0.5	23	12.5	"
12.30 P.M.	71	21.5	56.5	13.2	33.5	0.6	23	12.3	"
1.30	70.5	21.3	56	13	33	0.5	23	12.5	"
2.00	70	21	56	13	34 5	2	21.5	12	"

active movements become somewhat subdued when the animal is subjected to a rapid drop in environmental temperature during the first half hour, as indicated in the table, the fact that the body temperature remains at so conspicuously a high level would seem to indicate that the shock of the cold acts as a stimulus and is compensated for, perhaps nervously, by a sudden

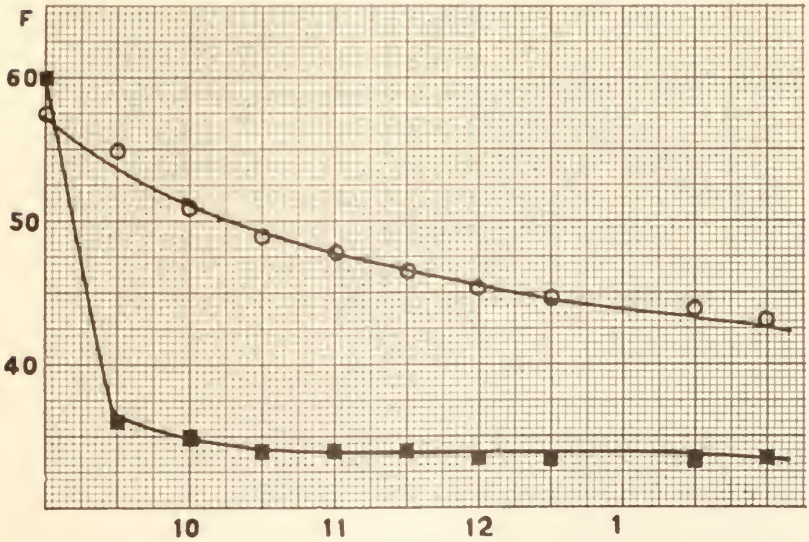

FIG. 2. Plot showing the average body temperature drop from seven painted turtles, correlated with a rapid environmental drop. The body temperatures are represented by circles, the environmental temperatures by squares.

increase in the production of heat. The temperature correlations from seven similar experiments are averaged and plotted against the time intervals and are shown in Fig. 2. Although the body temperature as a rule shows an immediate drop, it is not nearly commensurate with the rapid decline of the surrounding water, and it is conspicuous that during the succeeding four hours the differences are considerable and on the average remain from 8 to 10° F. above that of the environment.

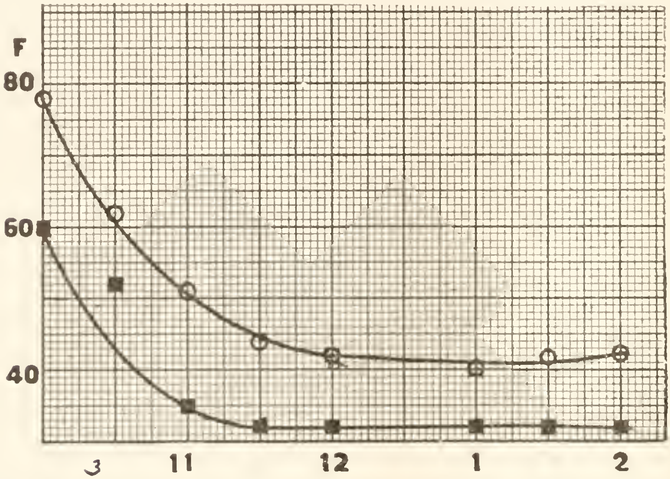

FIG. 3. Plot showing the average body temperature drop from five painted turtles, correlated with a slow environmental drop.

On the following and succeeding days, experiments with the same and other individuals of about equal weight were performed where the animals were subjected to slow cooling which extended over a period of two hours and were then maintained at the temperature of melting ice for another interval. Data from a typical experiment on a painted turtle, are shown in Table III., and the plot of the average fluctuations in temperatures from five records is given in the plot of Fig. 3.

From the table it is noted that at first the animals are quite active but this activity gradually merges into a period of quiescence as the temperature drops to about 45 degrees where, due probably to slight increase in activity, the drop is checked.

In the case of the body temperature changes in the snapping turtles less conspicuous differences are noted. They follow more

Table III.

Painted Turtle, Environmental and Body Temperatures;
Slow Cooling.

Time.	Room Temp.		Subj. Temp.		Env. Temp.		Diff.		Activity.
Aug. 2									
8.30	67° F.	19.5° C.	65.5° F.	18.5° C.	62° F.	16.5° C.	3.5° F.	2° C.	Active
9.00	72	22 2	60	15.5	52	11	8	4.5	"
9.30	75	24	55	12.5	47	8.5	8	4	Quiet
10.00	77.5	25.4	54	12	44	6.9	10	6	Slight activity
10.30	80.3	27	49	9.5	43	6	6	3	Quiet
11.00	81	27.5	45	7	36	3	9	4	"
11.30	82	27.9	43	6	33	0.5	10	5.5	Slight activity
12.00	83	28	42	5.5	33	0.5	9	5	Active
12.30	83	28	42	5.5	33	0.5	9	5	"
1.30	83	28	42	5.5	33	0 5	9	5	"

closely the external environmental drop than the painted form when cooled by either procedure. The chief point of interest in the comparisons is the location of the point of check in the drop which is five degrees lower on the average for the snapping turtle. No doubt these differences may be correlated to some extent, with the comparative differences in extent of soft body parts as compared to the harder parts in the two forms, but data on these points are not yet available. A typical protocol of the experiments with the snapping turtles is as follows. Four snappers were placed in containers at 10 o'clock on August 5th, in water drawn from the tap at a temperature of 60 degrees. They were subjected to slow cooling by the addition of ice to the outer container and records taken at intervals for four hours. In Table IV. are given data from specimen *C* of the series, weighing 582 grams and which is typical of them all.

Table IV.

Snapping Turtle. Environmental and Body Temperatures.

Time.	Room Temp.		Subj. Temp.		Env. Temp.		Diff.		Activity.
10.00 A.M.	81° F.	27° C.	78° F.	25.7° C.	60° F.	15.5° C.	18° F.	10.2° C.	Active
10.30	82	27.9	62	16.5	52	11	10	5.5	"
11.00	84	29	51	10.5	35	1.5	16	9	Quiet
11.30	85	29.5	44	6.7	32	0.0	12	6.9	"
12.00	86	30.2	42	5.5	32	0.0	10	5.5	Active
1.00	86	30.2	40	4.4	32	0.0	8	4.4	"
1.30	86	30.2	42	5.5	32	0.0	10	5.5	"
2.00	86	30.2	42	5.5	32	0.0	10	5.0	"

A similar series of experiments was performed on four specimens (*C*, *B*, *N* and *O*) on the following day and the average figures of these contribute to the plot, Fig. 4. The body temperature curve shows a comparable drop with that of the environment during the first hour and a half but is checked at about 40 degrees.

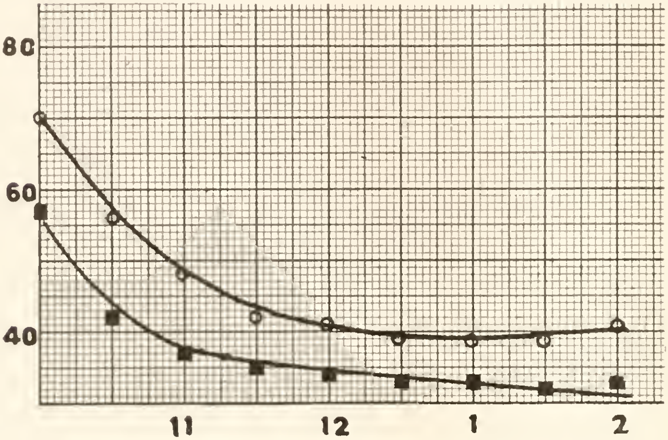

FIG. 4. Plot showing the average body temperature drop from four snapping turtles correlated with a slow environmental drop.

Body Temperature Fluctuations on Warming.—Two procedures were used in these experiments. On warm days with bright sunlight the animals were placed in containers either in air or in slight amount of water and put directly into the warm rays of the sun. In other cases the temperature of the containers was slowly raised by use of an alcohol lamp through a copper conducting unit. The results seemed not to differ greatly in either procedure. As a rule it was found that the animals could withstand gradual increase in temperature in air better than in water. This was due in part, no doubt, to slight though appreciable transpiration afforded in the former condition. As illustrative of the reactions of the painted variety to gradual increase in temperature, data from specimen *1* are given in Table V. This particular experiment together with others was carried out on an unusually favorable day, August 6. It was noted that hundreds of turtles were basking in the afternoon sun on old logs in the cove north of the laboratory, and it was thought that observations under experimental conditions might yield interesting although not exactly comparable results. The specimens were taken directly from the lake and fastened in containers in direct sunlight, one thermometer giving body readings and another, in air, the environmental changes. The surface temperature of the lake at this time was 20.5 degrees C. or 69 degrees F., and the initial temperatures of the turtle as they were taken, was only a few degrees less. But at the start of the experiment, after a lapse of some fifteen minutes, due to excitement and activity in the containers, it had approximated the air temperature.

TABLE V.

Time.	Subj. Temp.		Env. Temp.		Diff.		Activity.
Aug. 6							
1.45 P.M.	77° F.	25° C.	76° F.	24.5° C.	1° F.	0.5° C	Very active
2.00	78	25.7	79	26	1	0.3	" "
2.15	79	26	80	26.8	−1	−0.8	" "
2.30	92	33.5	106	41.2	−14	−7.7	Gasping, frothing, moisture about eyes. Active.
2.45	102	39	111	44.5	−9	−5.5	Gasping, frothing, small droplets about eyes.
3.00	99	37.3	109	43	−10	−6.3	Rapid breathing.
3.15	93	34	104	40.1	−11	−6.1	Active, gasping, rapid breathing.
3.30	90.1	32.5	95	35	−5	−2.3	Restless but frothing
4.00	80	26.8	80	26.8	0	0	Slowly moving

Whether or not the same relative increase in body temperature occurs in the case of the animals observed on the log, is of course, questionable. It was noted that the appendages were outstretched as well as the neck and that after an interval of an hour or more thus exposed, as a rule, a return to the water was made. No doubt also, some slight air currents played upon the body surface, thereby favoring any transpiration that might have occurred. Under experimental conditions, the operation of such factors to help keep the temperature down was very limited.

The average fluctuations in body temperature of five individuals are plotted against increased environmental temperature and shown in Fig. 5. It is noted there are points on the plot of the environmental curve which are somewhat widely displaced.

This is due to the fact that it is difficult to control the environmental temperature throughout the whole period, although in general, the trend of increase and decrease is fairly definite.

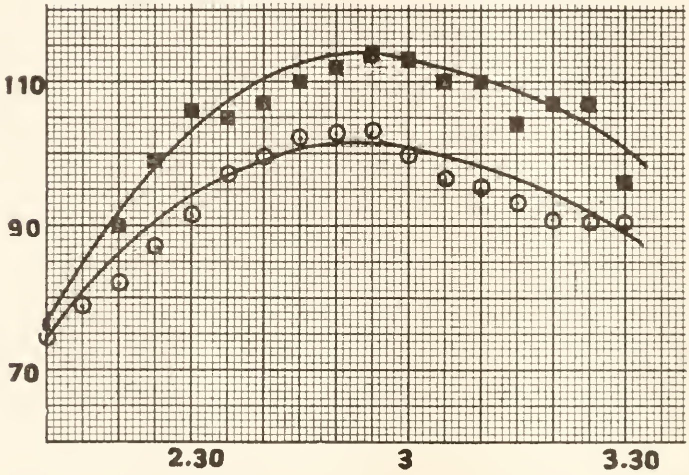

FIG. 5. Plot showing the average body temperature fluctuations of five painted turtles correlated with environmental rise.

The snapping turtle, in experiments under similar conditions, seems to indicate the same general trend, although these animals apparently do not increase in body temperature as rapidly, especially during the initial rise of environmental temperature as does the painted form. This slight difference, however, is transient if the increase in environmental temperature is steady, and eventually at the high critical points the body temperature as well as the reactions in this form simulate those in the other form. Data averaged and plotted from four individuals and checked against the environmental rise are shown in Fig. 6. It is noted on comparison with the preceding plot that increments of environmental temperature are not quite as effective in raising the body temperature in the snappers as in the painted variety. On anatomical grounds one is tempted to attribute these differences in part to a greater radiating surface of soft parts exposed in the snappers, for it is well known that the relative extent of the plastron in this form is considerably less than it is in the painted form. Whether there are in addition, physiological differences in the two forms can not at this time be stated on the basis of these experiments alone. The comparative behavior of the two forms taken as criteria, would certainly suggest that there might be. Thus the typical signs of discomfort, with rapid respiration and frothing about the mouth and the accumulation of moisture around the eyes appear at a body temperature, some ten degrees higher in the snapper than is the case with the painted turtle. At the high critical temperatures however, little differences in endurance could be noted in the two forms. From the limited data accumulated on this point, it appears that neither form withstands a body temperature maintained at between 102 and 105 degrees F. (39–41° C.) for thirty minutes or longer, and in the majority of individuals death results in a much less time.

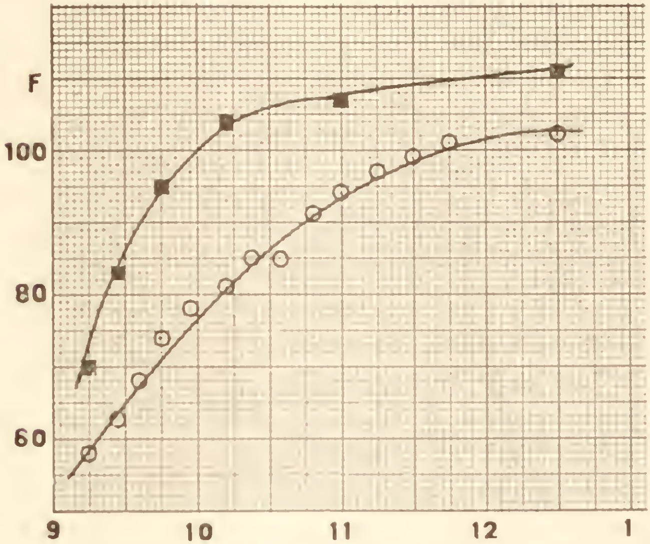

FIG. 6. Plot showing the average body temperature fluctuation of four snapping turtles correlated with environmental rise.

When placed in water as the surrounding medium and subjected to gradual increase in temperature animals of both groups are apparently incapacitated in their resistance. A typical experiment is cited in Table VI. This animal, a painted turtle, weighing 520 grams, was placed in a container of water at 9.30 on August 2. The water was drawn from the laboratory tap at practically room temperature and was heated during the extent of the experiment by a small alcohol flame through conduction. It is noted that the body temperature lagged slightly but kept pace with the environmental rise. At the end of two and one half hours, the temperature had reached the critical point and the animal was removed in a moribund condition from which it did not recover. Several other experiments with other individuals eventuated similarly.

TABLE VI.

Time.	Room Temp.		Subj. Temp.		Envir. Temp. (H_2O).		Diff.		Activity.
9.30	75° F.	23° C.	69.5° F.	21° C.	64° F.	18° C.	5.0° F.	+3.0° C.	Active
10.00	77.5	23.3	79	26.5	81	27.3	−3	− .8	Active
10.30	80.3	27	88	31.2	88	31.2	0	0	Very active
11.00	81	27.3	92	33.5	99	37.5	−7	−4	Scratching. Head outstretched above water.
11.30	82	27.9	104	40	108	42.5	−4	−2	Quiet, dead when removed.

Summary.—1. The rectal fluctuations of the common painted and snapping turtles of various sizes and weights are followed through ranges of non-critical and critical high and low environmental changes. In the non-critical range (50–80° F., 10–27° C.) in both forms the fluctuations are found to vary from 3 to 6 degrees F. (1.5 to 3° C.). When the environmental drop is rapid on cooling the rectal reading shows a somewhat greater lag than when cooled more rapidly. In both procedures a check in drop appears at about 40 degrees F., (4.5° C.) and there is maintained for a considerable interval of time.

2. Accompanying these temperature changes are noted differences in physiological activities, with muscular action at the outset which merges into a period of comparative quiet, this in turn followed by slow continuous movements.

3. Increase in environmental temperature is accompanied by a corresponding rise in body temperature and as a rule this is fatal if maintained at 102 to 105 degrees F., for any considerable

time, (30 minutes or more). At 80 degrees and above, animals show marked increased activity; signs of discomfort with rapid respiration; a frothing about the mouth and an accumulation of moisture upon the head and about the eyes.

4. In the absence of concrete data on comparative metabolic rate at different temperatures, these facts are tentatively interpreted to mean that there is in turtles a slight tendency to compensate for critical temperature changes in their environment.

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ON THE COLOR CHANGES IN THE SKIN OF THE LIZARD *PTYCHOZOÖN HOMALOCEPHALUM.*

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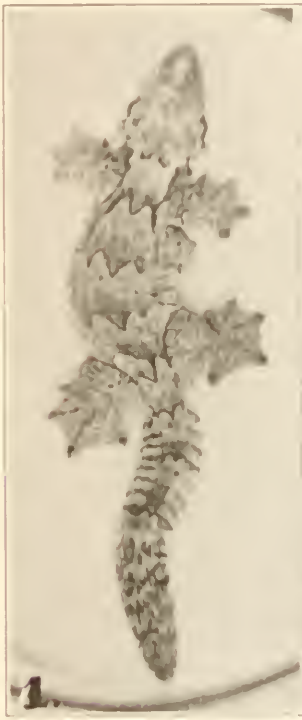
The javanese lizard *Ptychozoön homalocephalum* Crvdt. can change its color in some degree in connection with the difference in the color of its surroundings. In bright sunlight the dorsal surface of the animal is light gray with the exception of a number of pronounced blackish brown stripes which have a zigzag course in transversal direction and some spots of the same color at the lateral parts of its head and neck. In strongly shady surroundings the color of the skin between the transversal stripes darkens and assumes almost the same dark color as the stripes, which thereby become almost invisible. With these animals, which very commonly occur in 's Lands Plantentuin at Buitenzorg I have made some experiments to make out whether the change of color is controlled by the vision of the animals or if it is only due to the influence of light acting on the skin directly.

The experiments were made in August 1921 in the Treub-laboratorium at Buitenzorg with animals captured in the Plantentuin, in most cases by natives. I kept the animals in glass vessels with a diameter of about 20 cm., the bottom and sides of which were covered by white paper or black velvet. In these vessels they behaved quite calmly. The experiments consisted in the comparison of the color of the skin of the same animal (1) in a white-covered vessel, (2) in a black-covered vessel, and (3) in a white-covered vessel whilst its eyes were covered with a not transparent cap. The latter consisted of the cut-off digit of a rubber glove, in the top of which a small hole was cut. This cap was pushed over the head of the animal. It covered its eyes and the greater part of its head and neck, whilst the end of the snout was free and the respiration was not hindered. When the animal had been in the same conditions for some time (at least a quarter of an hour) I noted the color of the skin of its dorsal surface and then placed it in another vessel under different conditions.

Two of my animals I have photographed under the influences of the above-mentioned different conditions. From each of these six photographs were made on one morning, in the case of one of them in the following order: (1) head free, on white background, (2) head covered with cap, on white background, (3) head free, on black background, (4) head covered with cap, on white background, (5) head free, on black background, (6) head free, on white background. In a second series of photographs, taken from the other lizard, the succession of experiments was different, but with each exposure the conditions were different from those before and twice the animal was photographed under the same conditions. The photographs taken from animals in



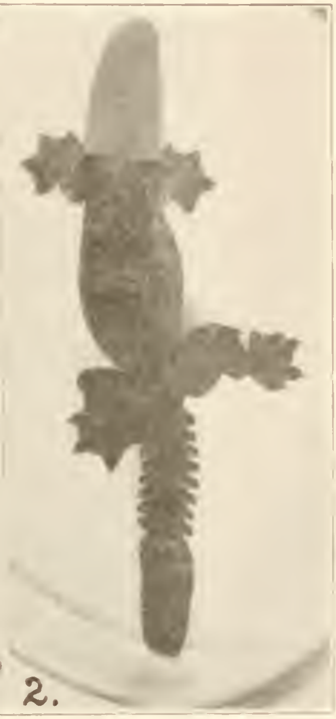


FIG. 1. *Ptychozoon homalocephalum*, on a white background. About ⅔ nat. size.
FIG. 2. *Ptychozoon homalocephalum*, the animal of Fig. 1, on a white background with covered head. About ⅔ nat. size.

the same conditions always gave the same results of the coloration of the skin.

As is shown by Fig. 1 the transversal stripes are very clearly visible when the animal with uncovered head is on a white background. The parts of the skin between these stripes have a light gray color which is brightest immediately behind the stripes and somewhat darker before them. A sharp contrast with this figure is that of Fig. 2. Here the animal is photographed on a white background, but with covered head. Now the color of the skin is almost uniformly dark gray whilst the transversal dark stripes are more indistinct. Almost the same color is assumed by *Ptychozoön* when it is surrounded by black (Fig. 3). Then also the stripes are only faint and the whole surface is almost uniformly dark gray.

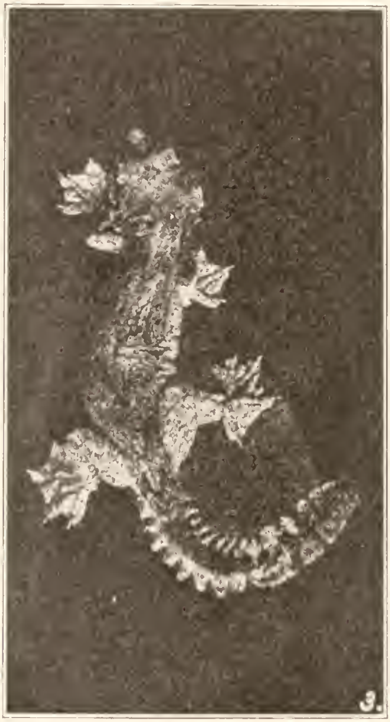


FIG. 3. *Ptychozoön homalocephalum*, another specimen than that of Figs. 1 and 2, on a black background. About $\frac{2}{3}$ nat. size.

I have made no experiments as to the length of time these lizards require for a complete change of color. Anyhow it takes less than a quarter of an hour. The photographs have been made with Ilford Panchromatic Plates, which were each exposed during one and a half minutes. The animals remained during this comparatively long time almost immobile on the same spot. The plates are developed in the same liquid during quite the same time. They have not at all been retouched or altered in any way. The positives are made with gaslight paper, those of Figs. 1 and 2 are exposed for quite the same time and have afterwards been worked out into a cliché together. Therefore these figures give reliable data for a comparison of the color of the skin of the same animal on a white background with free and with covered eyes. The technical peculiarities given above are mentioned to prove that the two figures give accurate data for the differences of the color under these different circumstances. Fig. 3 can only less directly be compared with the others, but it shows clearly enough that on a black background the color of the skin is of an almost even shade. The plate of this figure has been exposed for the same time as those of Figs. 1 and 2, but owing to the black background it is more or less underexposed. Therefore the animal appears brighter than it was in reality.

The external influences in these experiments were almost quite constant. I studied the color changes of *Ptychozoön* always on quite the same spot in diffuse daylight at a distance of circa 1 m. from the window. The *intensity of the light* was always practically the same, the time being the dry monsoon, when between 8 and 12 A.M. the intensity of the light in the tropics is quite equal. The difference in maximum and minimum of the *temperature* (if there was any difference at all) was very slight and also the *degree of humidity* did not vary noticeably during the experiments. The *structure of the substratum* was in all experiments the same. This, however, proved to be of little importance as was shown by a little minor experiment. Once I tried the same experiments by bringing the animals successively on white paper and on black velvet, but the color changes were the same as in the experiments with the animals in differently clad glass-vessels.

From the above stated peculiarities we may conclude that

Ptychozoön, when the external conditions are quite the same except the color of its surroundings, assumes the color which simulates in the highest degree that which it sees with its eyes. In other reptiles no instances are known that the changes of color are influenced by the vision of the animals, and also experiments on this question only gave negative results (cp. van Rynberk, 1906; Fuchs, 1914).

An important fact is moreover that the color of the skin of *Ptychozoön*, when the temperature is constant, becomes lighter on a white background and darkens on a black one. After Parker (1906) the pigment-cells of all reptiles expand in the light, whilst they contract in the darkness. In those cases, where by other authors different conclusions have been made, these would be due to changes of temperature which had not been taken into account by these authors. In those experiments in which the color changes of reptiles alternately in the shade and in the sunlight were studied, heat reactions may have influenced the movements of the chromatophores, in my experiments, however, the animals remained always on the same spot in diffuse daylight. The temperature was always very uniform and the lizards reacted directly on stimuli of white and black surroundings in the above described manner.

A contradiction to Parker's theory is found in the statements given by Thilenius (1897) for *Varanus*. According to this author *Varanus* assumes a dark color in the shade in 45–50°, whilst in the sunlight at a temperature of less than 30° the color becomes light. Parker has expressed his doubts as to the correctness of these temperatures, which might have been read from an ordinary mercury-bulb thermometer. With a more precise instrument the result would have been a much higher temperature. The difference between the true temperature and the one recorded in this case, however, would then have been more than 20°, and this difference is too large to be put on account of the inaccuracy of the instruments. In the case of *Ptychozoön* the color changes take place in the same way as described by Thilenius for *Varanus* and this proves that the chromatophores of at least some lizards contract in light and expand in dark.

In another way Fuchs (1914) has tried to explain the fact that light in some reptiles causes an expansion and in others a contraction of the chromatophores. Firstly he points to the fact that larvæ of amphibians show a different reaction to light in the different stages of development. In *Amblystoma* Babàk (1910) found that the very young larvæ react clearly on stimuli of light, becoming dark in the light and light in the shade. The eyes then have as yet no influence on the color changes. Afterwards, when the larvæ have grown older, the light has a completely other effect on the larvæ: the older larvæ become light in the light and assume a dark color in the dark. This reaction takes place under the influence of the eyes.

According to Fuchs the parietal organ, which in young larvæ is well-developed, has the function to impede the contraction of the chromatophores. Afterwards the eyes obtain an influence on the function of the pigment-cells, the illumination of the retina causes then a contraction of the chromatophores. Stimulation of the eyes therefore causes in older larvæ a reaction opposed to that caused by stimulation of the parietal eye. The older the larvæ, the stronger becomes the influence of the eyes as compared with that of the parietal eye. Consequently these animals become dark on a dark background and light on a light one.

In comparison with these different reactions towards light in young and older larvæ of amphibians Fuchs has put forward the hypothesis that in those reptiles, which show an expansion of pigment in light, the impeding influence of the functioning parietal organ is present. In those reptiles which show a contraction of pigment in light according to Fuchs either the parietal organ has lost its function in the course of phylogeny or ontogeny, or the eyes have acquired, as in older larvæ of amphibians, a regulating influence on the reactions towards light, on account of which the original reaction (expansion) was changed into the opposite (retraction).

In *Ptychozoön* no parietal eye is present. The general shape of the organs in this region of the brain is shown in Fig. 4. The epiphysis consists of a closed pouch which by means of a solid trace of cells is connected to the roof of the diencephalon just behind the commissura habenularis superior. It is directed backwards and covered by a protusion of the roof of the diencephalon, the "Zirbelpolster" of German authors. The well-developed paraphysis is found immediately before the latter. Almost quite the same arrangement and structure of these organs is found in *Platydactylus muralis* L., an allied species, in which the anatomy and development of the parietal organ is described at length by Melchers (1900). Besides that from the newly-hatched *Ptychozoön* (with a head-length of 11 mm.) a section of which is shown in the figure, another series of longitudinal sections was made from a younger stage, measuring 9 mm. from snout to occiput. The conditions found here make it highly probable that the development of these organs in *Ptychozoön* is quite the same as that in *Platydactylus muralis.*

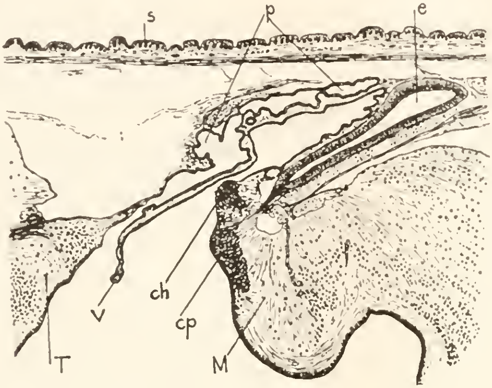


FIG. 4. *Ptychozoön homalocephalum*, newly-hatched specimen. Longitudinal section of a part of the brain. Hæmatoxylin-Delafield, eosin. × 43. *ch*, commissura habenularis superior; *cp*, commissura posterior; *e*, epiphysis cerebri; *M*, mesencephalon; *p*, paraphysis; *S*, skin; *T*, telencephalon; *v*, velum transversum.

In the case of *Ptychozoön* the above-cited hypothesis put forward by Fuchs agrees fairly well with the facts. The data available in the literature, however, are often in contradiction with this hypothesis. In *Platydactylus mauretanicus* the parietal organ is absent and yet this lizard assumes a dark color in the light and becomes light-colored in the dark. On the contrary *Stellio caucasicus* has a well-developed parietal eye and notwithstanding that the animal becomes light-colored in the light and dark in dark surroundings (cp. Studniczka, 1905, and Fuchs, 1914). These two instances already prove that there is insufficient evidence to uphold the above-mentioned hypothesis of Fuchs.

The microscopical structure of the skin in the lighter parts of the dorsal region of *Ptychozoön* differs in some respects with that in the dark stripes. In the light-gray parts (Fig. 5) the epidermis contains no pigment at all, the large pigment-cells consist of a large body which is situated in the cutis and a number of ramifications which are directed towards the epidermis and terminate immediately underneath the latter. In the living state a nearly continuous layer of black pigment is distributed under the epidermis by the contraction of the chromatophores. When the pigment-cells expand again, the pigment flows back to the deeper layer of the cutis and the color of the animal becomes much lighter.

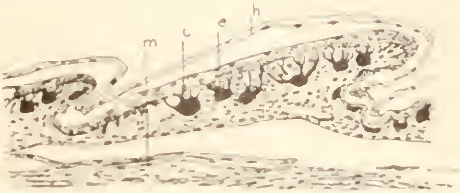


FIG. 5. *Ptychozoön homalocephalum*, newly-hatched specimen. Longitudinal section of a part of a light-colored portion of the dorsal region. Hæmatoxylin-Delafield, eosin. × 100. *c*, cutis with chromatophores; *e*, cell-layer of epidermis; *h*, horn-layer of epidermis, which has loosened from the parts underneath it; *m*, muscles.

In the dark stripes (Fig. 6) the chromatophores of the cutis are usually of a somewhat smaller size than those of the lighter parts of the body, the whole amount of cutis-pigment in a certain region, however, is about the same. Besides the cutis-pigment an epidermis-pigment is also present here. The uppermost parts of the cells of the epidermis are crowded with small black pigment granules which are found immediately underneath the

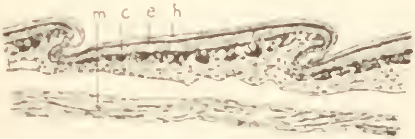


FIG. 6. *Ptychozoön homalocephalum.* Longitudinal section of a part of one of the dark stripes of the dorsal region of the specimen of Fig. 5. Hæmatoxylin-Delafield, eosin. × 100. *c*, cutis with chromatophores; *e*, cell-layer of epidermis, with epidermis-pigment; *h*, horn-layer of epidermis; *m*, muscles.

horn-layer of the epidermis. When the chromatophores of the cutis contract, the pigment of the epidermis remains on the same spot and the color of this region retains its black shade. It is only covered by a thin horn-layer and it is therefore much more conspicuous than the pigment of the chromatophores of the cutis, which is covered by the whole ectodermal layer.

The useful effect of these color changes in *Ptychozoön* is a matter of course. The lizards live on the trunk and larger branches of the trees. When they are in shady places they are hidden by their almost uniformly dark color. In the sunlight their light-gray hue equals that of the mossy background. They become still more inconspicuous by the possession of the queer zigzag dark stripes which procures them almost the same color and design as the bark of a tree overgrown with lichens.

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TOXICITY OF OXYGEN FOR PROTOZOA IN VIVO AND IN VITRO: ANIMALS DEFAUNATED WITHOUT INJURY.

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INTRODUCTION.

The fact that oxygen in an excessive amount is toxic for many if not all forms of life was first demonstrated by the numerous and very thorough experiments of Paul Bert ('74). But the swim bladders of some fishes normally contain oxygen at a pressure of 100 atmospheres (Haldane, '22). The cells lining the bladder apparently are acclimatized to the oxygen. On the other hand, Pütter ('05), working on the respiration of protozoa, states that parasitic forms, such, for instance, as *Opalina*, lived many days in a medium from which practically all the free (gaseous) oxygen had been removed. In view of the work of Pütter and that quoted by Haldane it occurred to me that a study of the toxicity of oxygen for the parasitic protozoa of many animals would be a worthy undertaking.

Wood-eating termites have the most abundant and the most varied entozoan fauna of all animals that have been studied.

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Approximately half the body weight of every worker and nymph, except for a few days after molting, is composed of many kinds of large, intestinal flagellates. Recently molted termites are lacking in pigment and for this reason may easily be distinguished from other individuals. There is no difficulty whatever, then, in being absolutely certain that all animals used in experiments harbor a teeming menagerie of protozoa; consequently termites lend themselves most admirably to many kinds of precise experimentation.

In a previous paper the writer ('25*b*) showed that *Trichomonas termopsidis* was entirely removed from the large Pacific Coast termite *Termopsis nevadensis* Hagen after 24 hours' oxygenation at one atmosphere pressure, and that all the other protozoa (*Trichonympha campanula*, *Leidyopsis sphaerica*, *Streblomastix strix*) were removed after 72 hours' oxygenation. The termites suffered no ill-effects *per se* from the oxygenation, although they died within three to four weeks after their protozoa had been removed. Recently this work has been carried further: four widely separated species of termites from two families have been used; and various intervals and amounts of pressure have been employed.

The bearing which these experiments have on the symbiosis between these termites and their intestinal flagellates will be reserved for a later paper. It is sufficient at present to say that the ability of these termites to live on their normal diet of wood is lost after their protozoa have been removed, regardless of the method employed in removing them.

Many other protozoa-harboring animals, such as cockroaches, earthworms, frogs and rats have been oxygenated. Some of the protozoa of the frog, rat, and man have been grown in cultures which have been oxygenated. And oxygenation experiments have been carried out on several free-living protozoa.

MATERIAL.

Of the termite family Rhinotermitidae, *Leucotermes tenuis* Hagen from British Guiana and *Reticulitermes flavipes* Kollar from Maryland were used; in the family Kalotermitidae, *Cryptotermes* sp. from British Guiana and *Termopsis nevadensis* Hagen from Oregon were used. Many thanks are due Dr. Alfred Emerson for *Cryptotermes* and *Leucotermes*.

Cockroaches (*Periplaneta americana?*) were obtained in Baltimore. So were all the parasitic protozoa in culture and most of the free-living protozoa. The exact source of the frogs (*Rana pipiens*), fish, salamanders and rats is not known.

METHODS.

The protozoa-harboring animals and the protozoan cultures were placed in flasks into which tank oxygen of 97 per cent. purity was run until the air was removed, or practically so. The flasks were then clamped down securely and from 1 (760 mm. mercury) to 2.5 standard atmospheres (except for temperature correction) were added to the 1 atmosphere at the time of the experiment. Other details of methods are given under the experiments on the different animals.

EXPERIMENTS.

1. *Termites.*

The effect of oxygenation at various pressures on the protozoa of four genera of termites is given in table I. The minimum time required to kill the protozoa of termites and other animals was obtained as follows: Several experiments were started at the same time so that any one could be stopped without interfering with

TABLE I.

TIME REQUIRED AT VARIOUS PRESSURES OF OXYGEN.

Pressure in atmospheres.	To Kill all Intestinal protozoa of										To Kill *Trichomonas* in Culture from			To Kill Host		
	Termites.								Cockroach.	Frog.	Frog.	Rat.	Man.	Termopsis.	Cockroach.	Frog.
	Rhinotermitidæ.				Kalotermitidæ.											
	Leucotermes.		Reticulitermes.		Termopsis.		Cryptotermes.									
	Hrs.	Min.	Hrs.	Min.	Hrs.	Min.	Hrs.	Min.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.	Hrs.
1 . . .	24		*		72		*									
1.5 .	4	30	9		9		7	30								
2 . . .	1	35	4		5		4	30								
2.5 .	1	15	1	40	2		1	55								
3 . . .		50		50	1	5	1									
3.5 .		30		30		40		35	3½	28	6	10	11	45	90	65

* Not killed in ten days.

the others, and the experiments were examined one by one until the minimum was found to be within certain limits. Then the experiments were set up again and were examined at various intervals within the known limit. Sometimes it was necessary to repeat the process many times, before a fairly accurate minimum was finally determined. The minimum thus determined was then tested out three times.

Termites were usually examined immediately after having been removed from oxygen and non-motility was the criterion for determining whether or not their protozoa had been killed. It was found that if a few protozoa were motile at the end of the oxygenation period they did not die later. Some hosts which had been freed of motile flagellates were examined at intervals up to ten days, and no protozoa ever appeared in any of them. It is of interest to note that the protozoa disappeared from the intestine very soon—usually within two to four hours—after they had been killed. They were probably digested by the termites.

At one atmosphere the protozoa of *Cryptotermes* and *Reticulitermes* were not all killed in all hosts in ten days; however, they were all killed in a few hosts even in three days. They were all killed in a great majority of hosts in ten days, but in a small number—perhaps about 5 per cent.—some protozoa were alive at the end of ten days.

The protozoa of *Leucotermes* were killed very much more quickly than those of the other termites until a pressure of 2.5 atmospheres was reached. These differences in oxygen toxicity are not correlated with size of termite hosts, for *Termopsis* is approximately twenty times as large as *Leucotermes* and ten times as large as *Cryptotermes*, but *Reticulitermes* and *Leucotermes* are about the same size. Difference in habit may be a factor, but *Reticulitermes* and *Leucotermes* are very similar in habit as well as in structure. The protozoa of these four termites, although all flagellates, are nevertheless quite distinct morphologically, many of them belonging to separate families. Hence, it is possible that the differences in oxygen toxicity may be found to be in the protozoa themselves.

In *Termopsis*, as was true in previous experiments (Cleveland, '25*b*), *Trichomonas* was killed first and *Trichonympha* last until a pressure of three atmospheres was reached. Then a peculiarly interesting result occurred, *Streblomastix* was killed before *Trichomonas* in many hosts. At a pressure of 2.5 atmospheres, *Trichomonas* and *Streblomastix* are both killed at or about the same time. Regardless of the amount of pressure, the largest protozoa of *Termopsis*, with the exception of one very small form (*Tritrypanoplasma*) not present in all hosts and abundant in only a few, are invariably the last ones to be killed, but in *Cryptotermes* and *Leucotermes* this is not always the case. In *Cryptotermes*, for instance, sometimes all individuals of the largest genus (*Diplonympha?)* are killed when many *Decescovina* are left alive.

Fifty individuals of *Termopsis* were confined in oxygen at 3.5 atmospheres. At the end of forty hours, every individual had become immobile. The experiment was stopped after forty-five hours, when it appeared that every individual was dead, although a few hours later two individuals became feebly motile, and were as active as ever two days later. Forty-five hours at 3.5 atmospheres must be about the time required to kill this termite, which is 67.5 times as long as it takes to kill its protozoa. In other words, oxygen at this pressure is 67.5 times as toxic for the protozoa of termites as for the termites themselves.

The time required to kill the protozoa of these termites at 3.5 atmospheres of oxygen certainly does not injure the termites in the least. Oxygenation at this pressure is surely a very rapid means of freeing termites of their protozoa. It is very much more satisfactory in many ways than incubation (Cleveland, '24), and furnishes a very ready means of determining whether or not all protozoa-harboring termites are dependent on their protozoa to digest their food for them.

In order to determine what effect, if any, partial pressures of other gases, particularly nitrogen, had on oxygen toxicity, termites of each of the four genera that were oxygenated were confined in five atmospheres of air (the partial oxygen pressure of five atmospheres of air approximates the total oxygen pressure of one atmosphere of oxygen) for the same time that they were confined in one atmosphere of oxygen. Five atmospheres of air, in every instance, gave exactly the same result as one atmosphere of oxygen. Thus, the toxicity of oxygen is unaffected by the presence of nitrogen and the rare gases of the air. In another experiment 3.5 atmospheres of air were used with the result that the protozoa were not killed at all. It is evident, then, that mere mechanical pressure does not kill the protozoa.

2. *Cockroaches.*

Since the protozoa of termites were so easily removed by oxygenation, it immediately became desirable to try the method on other protozoa-harboring insects. The cockroach has many protozoa and can be obtained easily in quantity. By pressing on the abdomen with the fingers or some mechanical instrument and forcing out some of the intestinal contents it is not difficult to determine just what protozoa an individual harbors, and the procedure does not injure the insect. Cockroaches with two ciliates, *Nyctotherus* and *Balantidium*, and two flagellates, *Lophomonas* and *Polymastix*, were oxygenated at 3.5 atmospheres. About 200 insects were used in these experiments. The minimum time required to kill all individuals of each of the four protozoan genera is given in Table II. It is interesting to note that the flagellates were both killed in 40 minutes, the same time required to kill the flagellates of the large Pacific Coast termite (*Termopsis*), at this pressure, while the ciliates were not all killed until 3½ hours, more than five times the time required to kill the flagellates living under identical conditions. From this it would appear that oxygen is actually more toxic for flagellate protozoa.

Table II.

Time Required at 3.5 Atmospheres of Oxygen to Kill All Individuals of Certain Intestinal Protozoa of

Frogs				Cockroaches		
No. of frogs used	Protozoa	Range in hours	Mean in hours	Protozoa	Hrs.	Min.
15	*Hexamitus*	3–7	5	*Lophomonas*		40
10	*Polymastix*	5–11	7	*Polymastix*		40
35	*Trichomonas*	8–15	12	*Nyctotherus*	3	30
30	*Opalina*	12–20	18	*Balantidium*	3	30
3	*Nyctotherus*	28	28			

Two other protozoa, an unidentified flagellate and *Endamœba blattæ*, were present in some of the cockroaches, but not in a sufficient number to make it feasible to work out the minimum time required to kill them. They were killed.

When cockroaches are confined in oxygen at 3.5 atmospheres, they are able to live approximately 90 hours, which is 26 times as long as their ciliate and 135 times as long as their flagellate protozoa live at this pressure. Here, again, as in termites, it is evident that the oxygenation which removes the protozoa of cockroaches certainly does little, if any, harm to the cockroaches themselves. They, unlike termites, live normally (indefinitely) after their protozoa have been removed. Oxygen, then, at 3.5 atmospheres is 135 as toxic for the flagellates and 26 times as toxic for the ciliates living in cockroaches as it is for the insects themselves.

3. *Earthworms.*

Many earthworms, harboring a fairly large number of ciliates of the genus *Hoplitophrya*, were oxygenated at 3.5 atmospheres, but the minimum time required to kill their protozoa was not determined. It is more than six and less than twenty hours.

4. *Frogs.*

After all protozoa had been removed from three invertebrates, it seemed highly desirable next to oxygenate a cold-blooded vertebrate harboring many protozoa. For this work the frog (*Rana pipiens*) was selected.

Most frogs harbor an abundant protozoan fauna; two ciliates, *Opalina* and *Nyctotherus*, and four flagellates, *Trichomonas* (*Tritrichomonas* in the opinion of some investigators), *Chilomastix*, *Hexamitus*, and *Polymastix*, are usually present in a fairly large number of hosts; in fact, all are sometimes present in the same host. Two hundred frogs were procured and just before being used in experiments each individual was examined in order to ascertain what protozoa were present. This examination was made by attaching a No. 7 hard rubber catheter, cut off at the insertion end to four inches in length, to a 5 cc. Luer syringe; by inserting the catheter into the rectum it was possible to draw out all or any amount of the rectal contents, which, when examined under the microscope, revealed immediately what protozoa each frog harbored. Of course, the number of protozoa present was also ascertained at the same time.

When frogs were oxygenated at 3.5 atmospheres, it was found that some of their intestinal protozoa were killed more quickly than others, so it became necessary to determine the minimum time required to kill all individuals of each protozoan genus, and in working this out it was noticed that the protozoa of a certain genus would be killed more quickly in one host than in another; consequently, in most instances, a fairly large number of hosts was used. The number of hosts used, the range in time and the mean in time required to kill the protozoa, are given in Table II. It has been impossible to think of a plausible explanation of why the protozoa of one frog are affected more adversely by oxygen than those of another. This was noticed when several frogs were oxygenated in the same flask at the same time. The same phenomenon was met in the oxygenation of termites and cockroaches. Perhaps more work will throw light on it.

It is interesting to note that it takes 28 hours to kill the ciliate *Nyctotherus* in the frog and 3½ hours to kill *Nyctotherus* in the cockroach. The flagellate *Polymastix* is killed in 40 minutes in the cockroach while the species of this genus that lives in the frog is not killed until approximately 7 hours. It would be most interesting, indeed, to cultivate *Nyctotherus* and *Polymastix* from both hosts and then subject them to the same oxygen pressure. It is very probable, though not certain, in view of the oxygenation studies of the frog *Trichomonas* in vivo and in vitro, that oxygen is actually more toxic for *Polymastix* and *Nyctotherus* in the cockroach.

Some frogs live as long as 65 hours in 3.5 atmospheres of oxygen, more than twice as long as their ciliate and five to six times as long as their flagellate protozoa.

Twenty tadpoles,[1] 2 with *Nyctotherus*, 3 with *Trichomonas*, 3 with *Opalina*, 4 with *Hexamitus*, and 8 with *Euglenamorpha*, were oxygenated at 3.5 atmospheres with the result that their protozoa were killed in approximately the same time as those of frogs. *Euglenamorpha* is not present in adult frogs, and it was primarily for this reason that tadpoles were oxygenated. This flagellate is very similar morphologically to plant-like free-living protozoa of the genus *Euglena* which, as will be seen later, must be oxygenated sixty five hours at 3.5 atmospheres before being killed, while

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[1] The method used to determine what protozoa each tadpole harbored was simple: Each individual was placed to itself in a small vesel; very soon a considerable quantity of fecal material was passed, which, when macerated and examined under the microscope, revealed the protozoa harbored.

Euglenamorpha is killed in approximately thirty hours. It is impossible to say whether this difference in oxygen toxicity is due to environment or metabolism. Here is an interesting situation, calling for careful investigation.

5. *Goldfish and Salamanders.*

After having removed all protozoa from an air breathing cold-blooded vertebrate, the frog, it seemed expedient to oxygenate a water breathing vertebrate harboring many protozoa. Goldfish and salamanders (*Necturus*) were used for this purpose. Twenty young goldfish, harboring large numbers of intestinal flagellates of the genus *Hexamitus*, and ten young salamanders, harboring large numbers of intestinal flagellates of the genera *Trichomonas* and *Prowazekella*, were oxygenated at 3.5 atmospheres. The same thing occurred here as in the oxygenation of frogs, namely, some hosts lost their protozoa sooner than others; *Hexamitus* in some goldfish was killed in 4 hours and in others not until 5 hours; *Trichomonas* and *Prowazekella* in some salamanders were killed in 9 to 10 hours and in others not until 11 to 12 hours. In adult hosts it would probably take slightly longer to kill all protozoa in all hosts. In the material used in this study all individuals of *Trichomonas* and *Prowazekella* were killed in all hosts in 12 hours and all individuals of *Hexamitus* in all hosts in 5 hours, while the hosts were not killed before 50 to 60 hours.

It is interesting to note how closely these death points of *Hexamitus* and *Trichomonas* inhabiting the water breathing vertebrates, salamanders and goldfish, parallel those of the *Hexamitus* and *Trichomonas* that inhabit the air breathing vertebrate, the frog (see Table II).

It is quite probable that the external parasitic ciliates of fish would be killed by oxygenation and without injury to the fish. These and other protozoan parasites, according to reports, do considerable damage to fish, which oxygenation would probably check.

What effect oxygenation would have on the hundreds of species of sporozoa in fishes should be determined.

6. *Rats.*

The next logical step in the development of the work was to oxygenate a warm-blooded vertebrate. *Trichomonas* (*Tritrichomonas* in the opinion of some investigators) is present in a fairly large number of rats; because of this, and owing to the ease with which rats may be obtained, the rat was selected. In order to demonstrate the presence of protozoa, fecal contents were removed by means of a catheter, as in the experiments with frogs, and were examined under the microscope.

It was found, however, that the rats themselves were not able to live more than five to six hours at 3.5 atmospheres of oxygen and that their protozoa were not killed in this time.

7. *Trichomonas from Frog, Rat and Man in Culture.*

Since it was impossible to remove the protozoa from a warm-blooded vertebrate by the method employed in removing them from the cold-blooded vertebrate, the problem of the toxicity of oxygen for the protozoa living in the intestines of rat and man was attacked in another manner, viz. the protozoa were grown in culture [1] and the cultures were oxygenated at 3.5 atmospheres by placing a few drops of the fluid from each culture in the same flasks that had been used in all the other experiments.

It was found (see Table I.) that the *Trichomonas* of frogs was killed in six hours, the *Trichomonas* of rats in ten hours, and the *Trichomonas* of man in eleven hours. Obviously, it is impossible, then, to kill the protozoa of the rat and of man by oxygenation at this pressure without killing the hosts themselves first.

It is interesting to note that the frog *Trichomonas* in culture is killed in about one-half the time required to kill it in the frog. This is perhaps explained in part by two facts: (1) oxygen is more soluble in water than in blood, and (2) the host furnishes some sort of resistance or barrier which makes it slightly more difficult for the oxygen to reach the protozoa.

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[1] Several of the culture media that have been employed by other investigators were used, but the following medium, which is largely a compilation from other methods, was found to be very satisfactory. For frog *Trichomonas*, sodium citrate 1 per cent, sodium chloride 0.5 per cent, Löffler's dehydrated beef serum 0.5 gram, distilled water 100 cc.; for *Trichomonas* of man and rat, 0.2 per cent, more NaCl was used. Growth was very abundant. Subcultures were made every three days of the organisms from rat and man. The frog *Trichomonas* lived three months sometimes without being transferred.

These experiments, as well as those on free-living protozoa, show that oxygen is directly toxic for protozoa, that it acts on them directly and not through any tissues of their hosts. In other words, the tissues of the hosts are not stimulated by the oxygen to give off products which kill the protozoa.

8. *Free-living Protozoa.*

It seemed highly desirable to compare the toxicity of oxygen for free-living protozoa with that for parasitic or entozoic protozoa; consequently, several genera of flagellates and ciliates were selected and were oxygenated in the same manner as were the cultures of parasitic protozoa.

TABLE III.

APPROXIMATE MINIMUM TIME REQUIRED AT AN OXYGEN PRESSURE OF 3.5 ATMOSPHERES TO KILL ALL INDIVIDUALS OF CERTAIN FREE-LIVING PROTOZOA.

Ciliates	Hours	Flagellates	Hours
Paramoecium	5	*Euglena*	65
Chilodon	4	*Heteronema*	50
Diophrys	60		
Holostica	50		

The results of these experiments are given in Table III. The fact that *Paramœcium* is killed in 5 hours, *Chilodon* in 4, *Holostica* in 50, and *Diophrys* in 60 shows conclusively that oxygen is just as toxic for some free-living ciliates as it is for some parasitic ciliates and flagellates. It is actually even more toxic for *Paramœcium* and *Chilodon* than for *Trichomonas* of frog, rat, and man in culture; the former are killed in 5 and 4 hours respectively, while it required 6, 10 and 11 hours respectively to kill the latter. And we have seen that the frog *Trichomonas* in culture is killed in about half the time required to kill it in the frog, yet it is killed much sooner than the ciliates, *Opalina* and *Nyctotherus*. It has not been possible to cultivate *Nyctotherus* and *Opalina* from frogs, but, reasoning from the time required to kill *Trichomonas* in vivo and in vitro, it would take 10 to 12 hours to kill them in culture, or more than twice the time required to kill *Paramœcium* and *Chilodon*. I cannot explain why oxygen is less toxic for *Diophrys* and *Holostica* than for *Paramœcium* and *Chilodon*. Perhaps a combined study of the metabolism, habitat, and oxygen toxicity of these and many other free-living ciliates will throw light on the problem.

After having oxygenated the ciliates, the results with the plant-like flagellates *Euglena* and *Heteronema* are not surprising. Some animal-like free-living flagellates would probably yield toxicity results quite similar to those obtained with parasitic flagellates.

SUMMARY AND CONCLUSIONS.

The toxicity of oxygen at various pressures for four genera of termites has been determined. At a pressure of 3.5 atmospheres the protozoa are all killed in two genera in 30 minutes, in one in 35 minutes, and in another in 40 minutes, while the termites themselves are not killed until 45 hours. Thus, oxygen is more than forty times as toxic for the protozoa as it is for the termites. This makes it possible to remove all protozoa from termites very easily and without injury to the host.

The protozoa of two termite genera were not killed at one atmosphere of oxygen even in ten days, while in two other genera they were killed in one and three days respectively. This gave an excellent opportunity to work out what effect, if any, partial pressures of other gases of the air, particularly nitrogen, had on oxygen toxicity. All four genera when confined in five atmospheres of air (partial O_2 pressure of 5 atms. of air approximates the total O_2 pressure of 1 atm. of O_2) gave exactly the same result as when confined in one atmosphere of oxygen for the same time. Thus, the toxicity of oxygen is in no way connected with or affected by the partial pressures of other gases of the air. It is the partial pressure of oxygen, and not mere mechanical pressure, that matters.

Cockroaches harbor many kinds of protozoa, all of which were removed by oxygenation at 3.5 atmospheres in $3\frac{1}{2}$ hours; the flagellates, *Lophomonas* and *Polymastix*, were killed in 40 minutes, and the ciliates, *Nyctotherus* and *Balantidium*, in $3\frac{1}{2}$ hours. The cockroaches themselves were not killed until 90 hours. Thus, oxygen at this pressure is 135 times as toxic for the flagellates and 26 times as toxic for the ciliates living in cockroaches as it is for the insects themselves.

It is highly probable that all insect-inhabiting protozoa may be removed by oxygenation without injury to their hosts. If so, the rôle which insects play in the transmission of protozoa from man to man, from animal to animal, from animal to man and from plant to plant can be worked out much more effectively. What effect, if any, oxygenation would have on other insect-transmitted organisms, bodies, inclusions, and agents would be well worth study.

Earthworms when oxygenated lose their ciliates and are uninjured by the process.

Frogs harbor many protozoa. More than 150 experiments have been carried out on the oxygenation of frogs, and all the intestinal protozoa may be removed without injury to the frogs. Table II shows the minimum time required to kill three flagellates, *Hexamitus*, *Polymastix* and *Trichomonas*, and two ciliates, *Opalina* and *Nyctotherus*. The ciliates are killed in less than one-half the time required to kill the frogs, and the flagellates in one-fifth to one-tenth the time.

The protozoa of two water breathing vertebrates, goldfish and salamanders, were all killed by oxygenation in less than one fifth the time required to kill their hosts.

If oxygenation will remove the protozoa of other amphibia, it will be possible to make some interesting studies on protozoal host specificity.

It is highly probable that all intestinal flagellates and ciliates may be removed from all invertebrates and from all cold-blooded vertebrates by oxygenation and that none of these hosts will be injured. It is also possible that the sporozoa, amœbæ, and blood-inhabiting protozoa may be removed from the same hosts in the same way and without injury to the hosts.

Many experiments have been carried out on *Trichomonas* from frog, rat and man in culture. All of these protozoa are killed by oxygenation (see table I for the minimum time), but the time required to kill them in all except the frog is longer than it takes to kill the host itself at the same pressure; so it is impossible to remove the protozoa from rats and human beings by confining them in oxygen at 3.5 atmospheres. Perhaps oxygen may be successfully administered to warm-blooded vertebrates in some other way. Work of this nature is in progress.

Oxygenation experiments have been carried out on four genera of free-living ciliates and two of free-living flagellates. Oxygen is certainly just as toxic for some free-living ciliates as it is for parasitic ciliates; for others, it is not. For *Paramœcium* and *Chilodon*, it is really more toxic; for *Diophrys* and *Holostica*, it is considerably less toxic. It is not very toxic for two plant-like flagellates, *Euglena* and *Heteronema*, but would probably be found to be just as toxic for some animal-like free-living flagellates as for some parasitic species.

Oxygen in excessive amounts is toxic for all animals, but protozoa possibly take up a correspondingly larger amount of it as the tension or pressure is increased than do higher animals and for this reason are affected more adversely than termites, cockroaches, earthworms and frogs. During oxygenation the protoplasm of the protozoa sometimes becomes very much vacuolated,[1] which may indicate that it is being consumed, perhaps actually burned up, by increased metabolism. However, the metabolism of higher vertebrates is said to be slowed down by increased oxygen pressure. But Amberson, Mayerson, and Scott ('24) were "able to show that the metabolic rate in some of the higher marine invertebrates, with well developed respiratory mechanisms, is closely dependent upon the oxygen tension in the water over a wide range."

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[1] It is also true that many dying protozoa, regardless of the cause of death, sometimes become vacuolated.

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